

**University of Lisbon**  
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**Dynamics and function of nicotinic acetylcholine receptors  
in the nervous system**

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To my parents.

To António.

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*O coração, se pudesse pensar, pararia.*

*Could it think, the heart would stop beating.*

Bernardo Soares, O Livro do Desassossego



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$\alpha 7$ KO	$\alpha 7$ -nAChR knockout animal
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AMPA	$\alpha$ -amino-3- hydroxy-5-methylisoxazole-4-propionic acid
AP	action potential
APC	adenomatous polyposis coli
BDNF	brain-derived neurotrophic factor
Biot	biotinylated
BrdU	5-bromo-2-deoxyuridine
$\alpha$ -Bgt	$\alpha$ -bungarotoxin
Ca <sup>2+</sup>	calcium
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	3'-5'-cyclic adenosine monophosphate
CG	ciliary ganglion
Ch	choline
ChAT	choline acetyltransferase
Cl <sup>-</sup>	chloride
CNS	central nervous system
COase	cholesterol oxidase
CREB	cAMP response element binding protein
CRIP1	cysteine-rich interactor of PD 23
D <sub>i</sub>	diffusion coefficients
DAG	diacylglycerol
DG	dentate gyrus
E	embryonic day
E <sub>Cl</sub>	chloride equilibrium potential
EB1	end binding protein 1
EC	entorhinal cortex
ECD	extracellular domain
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
FRAP	fluorescence recovery after photobleaching
GABA	$\gamma$ -aminobutyric acid
GCL	granule cell layer
GDP	giant depolarizing potentials
GFP	green fluorescent protein
Gly	glycine
GPCR	G-protein coupled receptor

GK	guanylate kinase
HF	hippocampal formation
IP <sub>3</sub>	inositol 1,4,5 triphosphate
K252a	tyrosine kinase inhibitor
KB	ketone body
KO	Knock-out
LTM	long-term memory
LTP	long-term potentiation
MAGUKs	membrane associated guanylate kinases
MAPK	mitogen-activated protein kinase
M $\beta$ CD	methyl- $\beta$ -cyclodextrin
mEPSC	miniature excitatory postsynaptic current
MMLV	Moloney's Murine Leukemia Virus
MSD	mean square displacement
MW	Mann-Whitney U
NA	Numerical aperture
nAChR	nicotinic acetylcholine receptor
nDBB	nucleus of the diagonal band of Broca
NMDA	N-methyl-D-aspartate
NGF	nerve-growth factor
NPC	neuronal progenitor cells
NMJ	neuromuscular junction
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
P	postnatal day
p75 <sup>NTR</sup>	pan-neurotrophin receptor
PBS	phosphate-buffered saline
PDZ	post synaptic density protein (PSD95)/Drosophila disc large tumor suppressor (DlgA)/ zonula occludens-1 protein (zo-1)
PI3K	phosphatidylinositol-3-OH kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC $\gamma$	phospholipase C, $\gamma$ subunit
PNS	peripheral nervous system
PSC	postsynaptic current
PSD	postsynaptic density
QDs	quantum dots
RFP	red fluorescent protein
RNAi	RNA interference
ROI	region of interest
RT	room temperature



SFK	Src-family tyrosine kinase
SGZ	subgranular zone
SH3	Src-homology 3
Shc	Src homology 2/-collagen-related protein
SSCs	spontaneous synaptic currents
STM	short-term memory
SPT	single particle tracking
Sub	subiculum
SV2	synaptic vesicle protein 2
t test	Student's t test
TM	transmembrane
trkA/B/C	tropomyosin-related kinase A/B/C
VGCC	voltage-gated calcium channels
VTA	ventral tegmental area
WT	wild-type



Nicotinic acetylcholine receptors (nAChRs) are broadly distributed in the nervous system. Due to the sub-cellular location and high diversity of nAChRs, they are thought to play a key role in setting the synaptic strength between neurons.

The present work aimed to study the mechanisms that regulate the fate of neuronal nAChRs on the cell membrane and clarify their role in the central nervous system.

I used single-particle tracking to follow surface nAChRs on neurons. Both heteropentameric  $\alpha 3$ -containing receptors ( $\alpha 3^*$ -nAChRs) and homopentameric  $\alpha 7$ -containing receptors ( $\alpha 7$ -nAChRs) access synapses by lateral diffusion, where they moved slower and showed a constrained behavior. The nature of synaptic restraints was receptor-dependent, since the disruption of either lipid rafts or PDZ scaffolds rendered half of the stationary  $\alpha 3^*$ -nAChRs to be mobile without changing the proportion of mobile  $\alpha 7$ -nAChRs.

I next investigated the acute action of brain-derived neurotrophic factor (BDNF) on the function of  $\alpha 7$ -nAChRs. Patch-clamp experiments showed that BDNF rapidly decreased  $\alpha 7$ -nAChRs-mediated responses in hippocampal interneurons. This effect was dependent on the activation of TrkB receptors, occurred through the phospholipase C/protein kinase C pathway and involved the actin cytoskeleton.

Finally, the role of  $\alpha 7$ -nAChRs on the adult neurogenesis was investigated. Stereotaxic retroviral injection into the dentate gyrus of wild-type and  $\alpha 7$ -knockout ( $\alpha 7$ KO) mice was used to label and birthdate adult-born neurons for morphological and electrophysiological measures; BrdU injections were used to quantify cell survival. In  $\alpha 7$ KO mice, adult-born neurons developed with truncated dendritic arbors and experienced a prolonged period of immature GABAergic signaling. Under these conditions, neurons received fewer synaptic inputs and were more prone to die during the critical period when adult-born neurons are normally integrated into networks.

Overall, the findings here reported support a regulatory role for  $\alpha 7$ -nAChRs in the nervous system, pointing out an important role of these receptors on synaptic transmission and plasticity in the brain.

**Keywords:** Nicotinic acetylcholine receptors; lateral diffusion; brain-derived

neurotrophic factor; adult neurogenesis; hippocampus

Os receptores colinérgicos do tipo nicotínico (nAChRs) são vastamente expressos no sistema nervoso. Na região do hipocampo, em particular, os mecanismos desencadeados por nAChRs influenciam processos de atenção, memória, aprendizagem, e constituem importantes alvos terapêuticos para doenças do fórum neurológico, neurodegenerativo e psiquiátrico.

Atendendo à sua distribuição sub-celular e à elevada diversidade de subunidades expressas no sistema nervoso central, estes receptores são geralmente associados a fenómenos de regulação da força sináptica entre neurónios. Ainda mais, quando activados, os nAChRs permitem a entrada directa e/ou indirecta de cálcio ( $\text{Ca}^{2+}$ ), elevando consequentemente a concentração citoplasmática deste ião. O aumento da concentração de  $\text{Ca}^{2+}$  citoplasmático, mesmo que transitória, activa várias e diferentes cascatas de sinalização que podem, inclusivamente, alterar a expressão génica. Por outro lado, se a entrada de  $\text{Ca}^{2+}$  for excessiva, são activados fenómenos de excitotoxicidade neuronal que eventualmente podem culminar em morte celular. Assim, a localização e função dos nAChRs são de extrema importância e consequentemente alvo de mecanismos de regulação nos neurónios.

O trabalho descrito nesta dissertação teve como objectivo estudar os mecanismos intracelulares que regulam o destino de nAChRs na membrana celular, assim como mecanismos que regulam a sua função, e por fim clarificar o papel dos nAChRs no sistema nervoso central.

Para estudar a localização e o tráfego dinâmico dos nAChRs em neurónios, recorri à técnica de *single-particle tracking SPT* com *quantum dots*. Dado que nesta técnica são estudadas partículas individuais, é possível distinguir diferentes tipos de nAChRs atendendo à mobilidade. Este estudo foi efectuado em culturas de neurónios de gânglios ciliares, que expressam dois subtipos de nAChRs: heteropentaméricos contendo a subunidade  $\alpha 3$  ( $\alpha 3^*$ -nAChRs) e homopentaméricos contendo a subunidade  $\alpha 7$  ( $\alpha 7$ -nAChRs). Os  $\alpha 3^*$ -nAChRs medeiam a transmissão sináptica nos gânglios ciliares, enquanto que os  $\alpha 7$ -nAChRs têm um papel regulador. Quanto à dinâmica dos nAChRs, identifiquei uma população imóvel e uma outra móvel, tanto para os  $\alpha 3^*$ -nAChRs como para os  $\alpha 7^*$ -nAChRs. Curiosamente, no caso dos  $\alpha 3^*$ -nAChRs, cerca de 70%

da população apresentava-se imóvel, enquanto apenas cerca de 30% dos  $\alpha 7$ -nAChRs se apresentava imóvel. Ambos os subtipos de nAChRs apresentavam movimento do tipo Browniano quando se deslocavam em áreas extrasinápticas e do tipo restrito quando em áreas sinápticas. Ambos os subtipos possuíam constantes de difusão superiores nas áreas extrasinápticas do que nas sinápticas. Distiguiu-se ainda, tanto para os  $\alpha 3^*$ -nAChRs, como para os  $\alpha 7$ -nAChRs, uma subpopulação móvel, que transita entre domínios sinápticos e extrasinápticos através de difusão lateral. Os valores de constantes de difusão foram semelhantes para os  $\alpha 3^*$ - e  $\alpha 7$ -nAChRs. Verificou-se, contudo, que a natureza das restrições sinápticas é diferente para os dois tipos de receptores. Por exemplo, perturbações ao nível das jangadas lipídicas ("lipid rafts") ou das proteínas do citoesqueleto contendo domínios PDZ aumentam a proporção de  $\alpha 3^*$ -nAChRs móveis para cerca de 70%, sem contudo alterarem a proporção de  $\alpha 7$ -nAChRs móveis. Curiosamente, os mecanismos que regulam a difusão dos nAChRs depende do ambiente celular. Por exemplo, o colesterol é importante para a difusão dos  $\alpha 7$ -nAChRs em neurónios de gânglios ciliares, mas não em neurónios de gânglios lombares.

A acção aguda da neurotrofina *brain-derived neurotrophic factor* (BDNF) na função dos  $\alpha 7$ -nAChRs foi também investigada. O BDNF é uma molécula que existe em grande abundância no hipocampo e que geralmente é libertada durante períodos de grande actividade neuronal. O BDNF foi inicialmente descrito como uma molécula de efeitos relativamente lentos, que ocorriam na escala de horas ou mesmo dias. Mais recentemente, foram descritas acções rápidas do BDNF em vários receptores ionotrópicos, o que me motivou estudar uma hipotética acção desta molécula sobre os  $\alpha 7$ -nAChRs. Este estudo foi efectuado em fatias de hipocampo de rato (3-4 semanas) preparadas agudamente. Utilizou-se a técnica de *patch clamp* para registar correntes iónicas geradas pela aplicação de agonistas dos  $\alpha 7$ -nAChRs. A amplitude máxima destas correntes foi usada como uma medida da activação dos  $\alpha 7$ -nAChRs. A aplicação exógena de BDNF inibiu rapidamente as respostas mediadas pelos  $\alpha 7$ -nAChRs expressos nos interneurónios do *stratum radiatum* da área CA1 do hipocampo. Este efeito é dependente dos receptores TrkB para o BDNF, ocorre através das vias da fosfolipase C/cinase C de proteínas e requer a activação de receptores de adenosina do tipo  $A_{2A}$ . A inibição dos  $\alpha 7$ -nAChRs não era dependente, contudo, da acção de cinases da família das Src. Demonstrou-se ainda que a regulação dos  $\alpha 7$ -nAChRs pelo par BDNF/receptor TrkB depende do

citoesqueleto de actina e está comprometida na ausência de  $\text{Ca}^{2+}$  extra- e intra-celular. Por fim, investigou-se o papel desempenhado pelos  $\alpha 7$ -nAChRs na neurogênese adulta. Nos mamíferos, a neurogênese no giro dentado mantém-se activa durante o adulto e parece ser essencial para o funcionamento do hipocampo. As células progenitoras dos neurónios expressam  $\alpha 7$ -nAChRs, o que despertou o interesse em investigar o papel destes receptores no desenvolvimento e maturação das células precursoras de neurónios. Injecções com 5-bromo-2-desoxiuridina (BrdU) permitiram observar que os neurónios dos animais *knockout* para o gene dos  $\alpha 7$ -nAChRs ( $\alpha 7$ KOs) são mais vulneráveis durante a fase de integração na rede neuronal que os neurónio de animais controlo. Esta fase crítica de morte neruonal ocorre entre a segunda e quarta semana após o início do processo de diferenciação e é determinante para a integração de neurónios na rede. De seguida, investigou-se o ritmo de desenvolvimento/maturação dos neurónios, na presença e ausência de  $\alpha 7$ -nAChRs. A injeção extereotáxica de retrovírus no giro dentado de animais adultos normais (controlos) e em  $\alpha 7$ KO foi utilizada para marcar e datar neurónios gerados no adulto, permitindo uma posterior análise morfológica (número de ramificações e comprimento das dendrites) e electrofisiológica (potencial de membrana, potencial de inversão do cloro, cinética das correntes ácido  $\gamma$ -aminobutírico (GABA)-érgicas, frequência e amplitude das correntes sinápticas espontâneas) destes neurónios. A análise das propriedades dos neurónios foi feita três semanas depois da injeção, quando os neurónios controlo apresentavam parâmetros morfológicos e electrofisiológicos característicos de neurónios maduros. Ainda mais, durante esta idade, os neurónios estão a atravessar o período crítico de integração no circuito. Verificou-se que, nos  $\alpha 7$ KOs, os neurónios gerados no adulto apresentavam-se menos diferenciados do que nos animais controlo, quer a nível morfológico, quer a nível electrofisiológico. Neste caso, os neurónios possuíam árvores dendríticas truncadas e menos complexas. Apresentavam também um prolongamento do período em que o neurotransmissor GABA actua como despolarizante. De facto, as próprias correntes GABAérgicas mediadas pelos receptores  $\text{GABA}_A$  apresentavam uma cinética característica de estadios imaturos. Em comparação com os neurónios controlo, apresentavam ainda uma menor frequência de correntes sinápticas espontâneas, que por sua vez possuíam também uma menor amplitude.

Em conclusão, identificaram-se, assim, novos mecanismos moleculares que controlam

o tráfego dinâmico dos nAChRs na membrana celular. Demonstrei, também, que estes mecanismos dependem da constituição dos receptores, do domínio subcelular e do tipo de célula. Verifiquei ainda que os  $\alpha 7$ -nAChRs são um dos alvos das acções rápidas do BDNF. Esta regulação poderá ser importante na regulação da transmissão e plasticidade sináptica no cérebro. Por fim, identificou-se um papel determinante dos  $\alpha 7$ -nAChRs na sobrevivência e no ritmo de desenvolvimento, maturação neuronal e integração dos neurónios durante a neurogénese no adulto. Em suma, os resultados apresentados nesta tese apontam para um papel fundamental dos nAChRs no hipocampo e são propostos vários factores intra e extracelulares que regulam o tráfego e função destes receptores nos neurónios.

**Palavras-chave:** Receptores colinérgicos nicotínicos; difusão lateral; *brain-derived neurotrophic factor*; neurogénese no adulto; hipocampo



**Chapter 1**



**GENERAL INTRODUCTION**



## GENERAL INTRODUCTION

### 1.1 SCOPE OF THE THESIS

The research presented in this thesis is aimed to clarify of the mechanisms that regulate the functional expression of neuronal nicotinic acetylcholine receptors (nAChRs) on the cell surface and elucidate about the role played by these receptors in the central nervous system (CNS).

### 1.2 CHAPTER OVERVIEW

Due to the broad distribution and diversity of nAChRs in the nervous system, as well as to the characteristics of nAChR-dependent signaling, these receptors are thought to play a key role in setting the synaptic strength between neurons. In the section 1.3 of **Chapter 1** some of the cellular and molecular mechanisms currently known to regulate synaptic strength will be revised. In section 1.4, nAChRs and nAChRs-mediated signaling will be described, stressing out the reasons why these receptors are in a favorable position to act as a neuromodulators of synaptic transmission in the CNS. In section 1.5, there is a general description of the neurotrophin brain-derived neurotrophic factor (BDNF), including some intracellular pathways activated by BDNF to induce rapid modifications in neurotransmitter receptors function. Finally, the “Objectives and Rationale” are summarized in section 1.6.

**Chapter 2** is about the *Materials and Methods* used in this thesis. In this chapter, the techniques used will be explained in detail, pointing out their advantages and limitations. Live imaging of quantum dots (QDs) and immunostaining of fixed cells were used to study the trafficking of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs. Patch-clamp experiments were done to 1) evaluate the acute action of BDNF on  $\alpha 7$ -nAChR function and 2) investigate the impact of  $\alpha 7$ -nAChRs in the development and integration of adult-born neurons

in the hippocampus. Stereotaxic retroviral injection into the dentate gyrus was used to label and birthdate adult-born neurons for morphological and electrophysiological measures; BrdU injections were used to quantify cell survival.

**Chapter 3** is the first chapter for the *Results* section. Real-time imaging with QDs was used to study the lateral diffusion of neuronal  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs. These experiments were executed in dissociated chick ciliary ganglion neurons. We started by characterizing the mobility of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs in this system. Both receptors could access synaptic domains by lateral diffusion, displaying Brownian motion in extrasynaptic space and being constrained and move more slowly in synaptic space. The nature of their movement restraints was, however, different for  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs; lipid rafts, PDZ-containing scaffolds, microtubules, and actin filaments differentially affected their mobility. We found that control of nAChR lateral mobility, therefore, is determined by mechanisms that are domain-specific, receptor subtype-dependent, and cell-type constrained. The outcome is a system that could tailor nicotinic signaling capabilities to specific needs of individual locations.

**Chapter 4** will focus on the acute action of the neurotrophin BDNF on the function of  $\alpha 7$ -nAChRs. Patch clamp experiments were performed in fresh hippocampal slices taken from young rats. Acetylcholine- or choline-evoked currents were recorded in CA1 interneurons and were used as a measure of  $\alpha 7$ -nAChR function. BDNF rapidly reduced the amplitude of  $\alpha 7$ -nAChR mediated currents when applied in the perfusion solution. This effect was dependent on phospholipase C/protein kinase C signaling pathway and required  $\text{Ca}^{2+}$  as a cofactor. The present findings disclose  $\alpha 7$ -nAChR as a novel target for rapid actions of BDNF that might play important roles on synaptic transmission and plasticity in the brain.

**Chapter 5** will elucidate about the functional relevance of  $\alpha 7$ -nAChRs in the adult hippocampal formation (HF). In these experiments, adult mice were stereotaxically injected with Moloney murine leukemia virus-green fluorescence protein (MMLV-GFP) into the dentate gyrus to label and birthdate adultborn neurons in vivo; BrdU injections were used to quantify cell survival. In  $\alpha 7$ -nAChRs knockout mice, we observed a

reduced survival, delayed maturation and deficient integration of adult-born neurons in the network. This evidence points to a critical role of  $\alpha 7$ -nAChR in the fate of newborn dentate granule neurons.

This thesis will end with **Chapter 6**. A synopsis of all major findings is given. Furthermore, the possible consequences and implications of the new insights gained in the present study will be given and future perspectives for the study of nAChRs in the nervous system will be discussed.

**Chapter 7** includes all the references used in this dissertation.

### **1.3 NEURONAL TRANSMISSION AND SYNAPTIC STRENGTH**

#### **1.3.1 Neuronal communication: evolution of the nervous system**

The survival of an organism relies on the ability that cells have to communicate between each other and generate a global and effective response facing the changes that occur in the environment. When one looks at the evolutionary scale, the more evolved an organism is, the more complex is its nervous system and the more sophisticated is its behavior. But despite the large behavioral differences found in behavior among species, comparative studies of both vertebrates and invertebrates have revealed that brains evolved rather conservative compared with other morphological structures. Likewise, vertebrates, from lampreys to humans, are strikingly similar in the overall brain organization. In the last century, electrophysiological, pharmacological and molecular studies have provided a global understanding of the fundamental mechanisms of neuronal communication, which were also found to be highly conserved across a range of animal species. Despite it, changes in neural connections, neurotransmitters and membrane properties have occurred frequently in evolution. Thus, the brains of animals are a combination of small novelties that appear against a background of conserved features.

In general, neural networks evolved to regulate an organism's internal environment

and maintain its homeostasis. Consequently, the network developed in a way to sense perturbations upon the endogenous and exogenous systems, appropriately respond to these perturbations. Moreover, neural networks have the ability to form a memory of any particular episode in a way that allows them to respond more efficiently on the next time the same episode occurs. The capacity of the neural activity generated by an experience to modify neural circuit function and thereby modify subsequent thoughts, feelings, and behavior is generally called neuronal plasticity. Information storage and plasticity require that synapses carry out two opposing tasks: maintaining stable long-term synaptic connections while at the same time remaining plastic and allowing for rapid changes in synaptic strength. A major challenge has been to understand how the large array of synaptic proteins that govern these opposing processes are regulated to selectively establish, maintain, and modify the strength of synapses.

### **1.3.2 From the neuromuscular junction to the brain**

As an emergent structure, some of the properties of the nervous system can be explained by the low-level properties of units in the context of their interactions. It is now consensual that the synaptic function is the basic and universal property of neural circuits. The simplest type of synapse that one can find in nature is the neuromuscular junction (NMJ). In contrast to synapses in the CNS, the NMJ connects two different cell types, the neuronal cells and muscle cells. By the simplicity of the NMJ and due to its easy technical accessibility, it become one of the most important model systems in synapse research and was the base for the molecular principles of neurotransmission. Essentially, the function of the NMJ, as any chemical synapse, consists in the conversion of an electric signal conducted by the presynaptic cell to a chemical signal, which can be perceived by the target cell.

Though NMJ and neuron-neuron synapse share the basic mechanisms of synaptic transmission, there are clear differences between both types of synapses. In both cases, the electrical signal [or action potential (AP)] induces depolarization of the presynaptic terminal membrane to the extent of opening voltage-gated calcium channels (VGCC). The transient increase of the local concentration of cytoplasmic  $\text{Ca}^{2+}$  induces the fusion neurotransmitter-containing vesicles with the presynaptic membrane and,

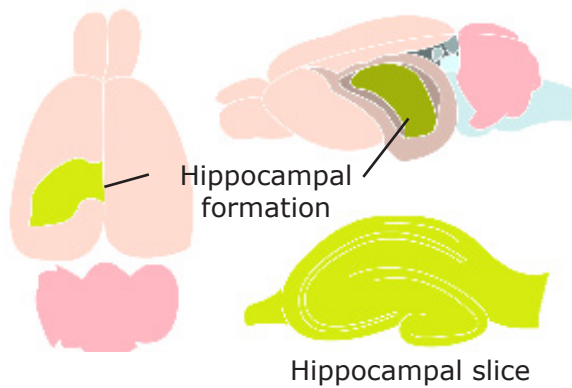
consequently, stimulates the release neurotransmitters from the active zone into the synaptic cleft. The neurotransmitters selectively bind to neurotransmitter receptors located in the postsynaptic density (PSD) of the neighboring cell, opening their internal pores and allowing the influx of ions. This inward current eventually generates a new AP, leading to the propagation of the electric signal, which spreads within the muscle cell.

While in the NMJ, neurotransmitter vesicles are always loaded with acetylcholine, in neuron-neuron synapses, the type of the neurotransmitter released can vary, depending on several factors (e.g. cell type, developmental stage of the cell). The endplate of the NMJ comprises only two types of nAChRs, while the neuronal PSD can express panoply of neurotransmitter receptors, which play either excitatory or inhibitory actions.

The molecular/structural differences found between NMJ and neuron-neuron synapses can be easily understood if one looks at their jobs assignments. The synaptic contact at the muscle end plate is optimized to propagate and amplify a single AP in the muscle with maximal reliability. In neuron-neuron synapses, the focus lies less on the transmission of a single AP but rather on integration of multiple signals within the neuronal network and on the complexity of the network itself. In the CNS, a single neuron gets input from several thousands of other neurons. In contrast to the simple architecture of motor neurons, the complex organization of neuronal networks demands regulatory mechanisms orchestrating excitatory actions.

### **1.3.3 Synaptic transmission in central synapses – Hippocampus as a model system**

Synaptic transmission is the basis of most nervous system function, including controlling body parts, memory, learning and cognition. Complex and highly regulated steps take place at both pre- and post-synaptic components to guarantee normal neuronal communication. The hippocampal formation (Fig 1.1; HF) is probably one of the brain regions more extensively studied in the CNS due to its central role learning and memory. Furthermore, the anatomical structure of HF allows one to cut thin slices out of the HF in a way that preserves all of the major connections. For these reasons, HF has been



**Figure 1.1 – A simplified diagram of the hippocampus in the brain and an hippocampal slice.**

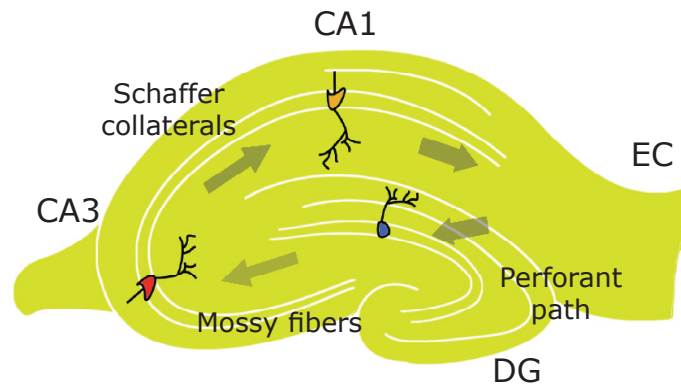
the primary model for transmission in central synapses.

The rodent HF is a C-shaped structure that is situated in the caudal part of the brain. Three distinct subregions can be distinguished: the dentate gyrus (DG), the hippocampus proper (consisting of CA3, CA2 and CA1) and the *subiculum* (Sub) (Amaral and Lavenex, 2007;

Burwell and Witter, 2002). The cortex that forms the HF has a three-layered appearance (for a review, see Förster et al., 2006). The first layer is a deep layer, comprising a mixture of afferent and efferent fibres and interneurons. In the DG, this layer is called the *hilus*, whereas in the CA regions it is referred to as the *stratum oriens*. Superficial to this polymorph layer is the cell layer, which is composed of principal cells and interneurons. In the DG this layer is called the granule layer, whereas in the CA regions and the *subiculum* it is referred to as the pyramidal cell layer (*stratum pyramidale*). The most superficial layer is referred to as the molecular layer (the *stratum moleculare*) in the DG and the *subiculum*. In the CA region, the molecular layer is subdivided into a number of sublayers. In CA3, three sublayers are distinguished: the *stratum lucidum*, which receives input from the DG; the *stratum radiatum*, comprising the apical dendrites of the neurons located in the *stratum pyramidale*; and, most superficially, the *stratum lacunosum-moleculare*, comprising the apical tufts of the apical dendrites. The lamination in CA2 and CA1 is similar, with the exception that the *stratum lucidum* is missing in CA1.

The principal neurons of the different HF subfields are interconnected via the excitatory trisynaptic circuit (Fig 1.2; Witter and Amaral, 2004; van Strien et al., 2009). According to the canonical model, the first step of the trisynaptic HF pathway is formed by a unidirectional projection from the DG to CA3: the mossy fibres. The Schaffer collaterals, which originate in CA3 and project to CA1, are the next step in the polysynaptic loop. Finally, CA1 send their projections to the Sub and deep layers of entorhinal cortex (EC), while Sub cells send their projections mainly to EC.





**Figure 1.2 – The neural circuitry in the rodent hippocampus.** An illustration of the hippocampal circuitry. The traditional excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus (DG)–CA3–CA1–EC) is depicted by solid arrows. The axons of layer II neurons in the entorhinal cortex project to the dentate gyrus through the perforant pathway (PP). The DG sends projections to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. The DG cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells.

The net flow of information in the HF is strongly modulated by the action of the local-circuit inhibitory interneurons, whose cell bodies are distributed throughout all layers of the HF. Although interneurons are poorly represented in the HF and comprise ~10-15% of the total neuronal population, they exert a powerful control on network excitability and information processing in the brain since a single inhibitory nervous cell may contact 1000-3000 pyramidal cells via extensive arborization (Li et al., 1992; Buhl et al., 1994a). For this reason, interneurons can phase the output of principal cells giving rise to a coherent oscillatory activity (Klausberger et al., 2003; Klausberger et al., 2004; Klausberger and Somogyi, 2008), which has been implicated in encoding, consolidation and retrieval of information in the hippocampus (for review, see Freund and Buzsaki, 1996). Because of their central role in pacing, timing and synchronizing neural circuits in both spatial and temporal domains, knowledge on the mechanisms that control and/or modulate interneuronal function will be crucial to understand hippocampal computation.

Different types of interneurons appear to perform specific and diverse functions in the hippocampus (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008). For example, some interneurons potentially inhibit pyramidal cells by acting directly on their

cell bodies or axon hillocks (Gulyás et al., 1993; Buhl et al., 1994b; McBain et al., 1994; Sik et al., 1995; Miles et al., 1996), whereas others inhibit pyramidal cell activity at their dendrites (Han et al., 1993; Gulyas et al., 1993a,b; Sik et al., 1995; Miles et al., 1996). Another group of interneurons specifically inhibit other interneurons (Acsady et al., 1996; Gulyas and Freund, 1996; Hajos et al., 1996; Blasco-Ibanez et al., 1998). Some interneurons also appear to show long-range projections that cross the sub-area border and are involved in the coordination of spike timing across sub-areas (Sik et al., 1994, 1995). The large variety of inhibitory cells in the hippocampus explains why it has been so complicated to find common properties that would allow grouping them in different subtypes. Most if not all hippocampal interneurons produce the neurotransmitter  $\gamma$ -aminobutyric acid (GABA). Choline acetyltransferase (ChAT), the synthesizing enzyme of acetylcholine, is localized in a small number of interneurons in the CA1 region of the hippocampus and the dentate gyrus of the rat (Frotscher et al., 1986), but even these cells are also likely to contain GABA. According to Somogyi and Klausberger, there are at least 16 different types of interneurons just in the CA1 hippocampal area. It is currently thought that this high heterogeneity of interneurons is essential for shaping different patterns of activity in the neural network. Even from a minimalist point of view, at least 10 types of distinct interneurons innervate a single pyramidal cell and actually it is not known yet if all pyramidal cells are uniformly innervated. In addition, the type of inhibition played by a single interneuron in the network can change over time depending on the afferents that activate it (Croce et al., 2010). Finally, the intrinsic passive and active electrical properties of interneurons, their synaptic kinetics and the subcellular domains of the target neurons on which they make GABA-releasing synapses are also important for the impact that they can exert in the network (Morin et al., 1996).

#### **1.3.4 Synaptic plasticity – basis for learning and memory?**

The activity-dependent modifications of the efficacy of synaptic transmission (or synaptic strength) at preexisting synapses are generically referred as synaptic plasticity. A key concept is that synaptic strength is bidirectionally modifiable by different patterns of activity, this meaning that synapses can be potentiated or depressed depending on

the input. It has been proposed that synaptic plasticity plays a central role in the early development of neural circuitry and in the capacity to incorporate transient experiences into persistent memory traces (although the molecular foundations subjacent to these two processes might differ due to different requirements).

It is widely accepted that synaptic plasticity requires structural changes that occur too quickly to be accounted for by nuclear or even dendritic protein synthesis (Kasai et al., 2010). Later, these changes must be stabilized or consolidated in order for memory to persist (Harris et al. 2002). The temporary reversible changes are referred as short-term synaptic plasticity [or short-term memory (STM)], while the persistent changes are called (long-term synaptic plasticity [or long-term memory (LTM)]). Numerous forms of STM, lasting on the order of milliseconds to several minutes, have been observed at virtually every synapse examined in organisms ranging from simple invertebrates to mammals (Zucker and Regehr, 2002). These are thought to play important roles in short-term adaptations to sensory inputs, transient changes in behavioral states, and short-lasting forms of memory. Repetitive activation of excitatory synapses in the hippocampus can cause a potentiation of synaptic strength that could last for hours or even days (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). This phenomenon, termed long-term potentiation (LTP), has been the object of intense investigation because it is widely believed that it provides an important key to understanding some of the cellular and molecular mechanisms by which memories are formed (Martin et al, 2000; Pastalkova et al, 2006; Whitlock et al, 2006).

#### **1.3.4.1 Mechanisms subjacent to modifications on synaptic strength in the CNS**

To understand the mechanisms that underlie synaptic plasticity, one should look in detail at synapses since they are considered the first level of organization beyond the molecule. Moreover, the molecular mechanisms that orchestrate or mediate synaptic structural changes often play a role in synaptic plasticity and memory.

The regulation of synaptic strength at central synapses is dependent on many factors (Sudhof and Malenka, 2008). At the presynaptic level, two principal points of regulating

transmitter release exist: (1) the peak  $\text{Ca}^{2+}$  concentration produced by an action potential, i.e., the conversion of an action potential to a  $\text{Ca}^{2+}$  current (Katz, 1969); and (2) the release probability per given  $\text{Ca}^{2+}$  concentration, i.e., the conversion of a  $\text{Ca}^{2+}$  signal to exocytosis (Perin et al., 1990). Intracellular messengers and extracellular modulators released by neurons and glia can influence the effectiveness of an action potential to evoke transmitter release by affecting at least one of the regulation points described. Moreover, the effectiveness of release can undergo sustained activity-dependent changes over time. For example, signaling back to the presynaptic terminal from the postsynaptic neuron has short-term effects on transmitter release and can even induce a number of forms of long-lasting synaptic plasticity (Fitzsimonds and Poo, 1998).

Besides the complex presynaptic machinery guaranteeing tuned neurotransmitter release, the PSD determines neurotransmitter response and responsiveness. Recent advances in high-resolution electron microscope tomography coupled with specific antibody labeling have allowed visualizing the anatomical structures of PSDs directly (Chen et al., 2008). The majority of these studies have been done at glutamatergic synapses. The first layer of a PSD mainly contains membrane receptors, ion channels and transmembrane cell-adhesion molecules, with N-methyl D-aspartate (NMDA) receptors at the centre and  $\alpha$ -amino-3- hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors at the periphery. The second layer is enriched with scaffold (or adaptor) proteins, which are closely coupled to the membrane receptors and ion channels and are arranged perpendicular to the PSD membrane. The third layer is comprised of scaffolds proteins that bind to other scaffold proteins and are arranged in parallel to the PSD membrane. The proteins in this third layer are further connected to the actin cytoskeleton. All of these membrane receptors and scaffold proteins form a protein network to which other cytoplasmic proteins and enzymes can bind.

The PSD is responsible for adjusting type, number, localization and properties of neurotransmitter receptors. In the CNS, the majority of fast excitatory input is mediated by glutamatergic AMPA, kainate, and NMDA receptors, while inhibitory transmission is governed almost entirely by glycine (Gly) receptors and  $\text{GABA}_A$  receptors. The main

structure of these receptors is conserved, supporting the hypothesis of a common evolutionary origin (Chiu et al., 1999). However, in contrast to the structural similarities, there are pronounced functional differences among ligand-gated ion channels; the receptors are sensitive to different ligands and exhibit different ion selectivity. AMPAR and NMDAR are activated by glutamate and permeable for cations, inducing excitation in a neuron. GABA<sub>A</sub>R and GlyR are activated by GABA and glycine respectively, playing inhibitory roles since they allow chloride (Cl<sup>-</sup>) ions to enter the cell and, consequently hyperpolarizing the membrane.

#### **1.3.4.2 Trafficking of neurotransmitter receptors – exocytosis, lateral diffusion and endocytosis**

Each type of receptor has its own complementary accessory proteins and regulatory elements, but there are some interesting commonalities between excitatory and inhibitory receptors of the CNS. Because neurons are highly arborized cells, newly synthesized receptors have to travel long distance along neuritis to reach the most distal synapses. This can be achieved either by vesicular transport from internal pools as well as by lateral diffusion on the cell membrane (Cognet et al., 2006; Newpher and Ehlers, 2008). These mechanisms of receptor delivery to synapses were first described for acetylcholine receptors in the NMJ (Axelrod et al., 1976; Anderson and Cohen, 1977) and have since been found to occur similarly for glutamate (Borgdorff and Choquet, 2002), GABA (Pooler and McIlhinney, 2007) and glycine (Meier et al., 2001) receptors in the CNS. In fact, receptor trafficking in the CNS has been particularly well documented for glutamate AMPA receptors, but it was shown to occur for most of receptor types. The overall number of receptors is adjusted by the rate of protein synthesis, rate of internalization and integration from and to the membrane, respectively. Receptors are synthesized at the endoplasmatic reticulum and processed at the Golgi apparatus. Insertion sites seem to be distinct for different receptors types. In all cases, and once in the cell membrane, receptors are stabilized and anchored through different mechanisms, involving transient specific binding to scaffold molecules, steric repulsive interactions with other transmembrane molecules such as other receptors, and interactions with synaptic adhesion molecules (Heine et al., 2008a) and submembrane

cytoskeletal fences (Allison et al., 1998).

The relatively high concentration of neurotransmitter receptors within the postsynaptic specialization and the interactions between these receptors and intracellular scaffold proteins both led to the predominant view that synaptic receptors are tightly fixed within the synapse. However, evidence for dynamic receptor populations and receptor exchange at synapses has long existed (Axelrod et al., 1976; Anderson and Cohen, 1978). More recently, single-particle tracking of glutamate receptors in the postsynaptic membrane has demonstrated that glutamate AMPARs rapidly alternate between periods of Brownian-like lateral mobility, often at extrasynaptic sites, and periods of confinement, mostly at synapses (Borgdorff and Choquet, 2002). The Brownian movement depends on thermal agitation of molecules (in this case, lipids shaking on proteins) and is characterized by a linear relationship between the surface explored versus time. The confinement at synapses results of molecular crowding and the presence of receptor binding sites at the synaptic scaffolds, which promote the concentration of receptors at the PSD.

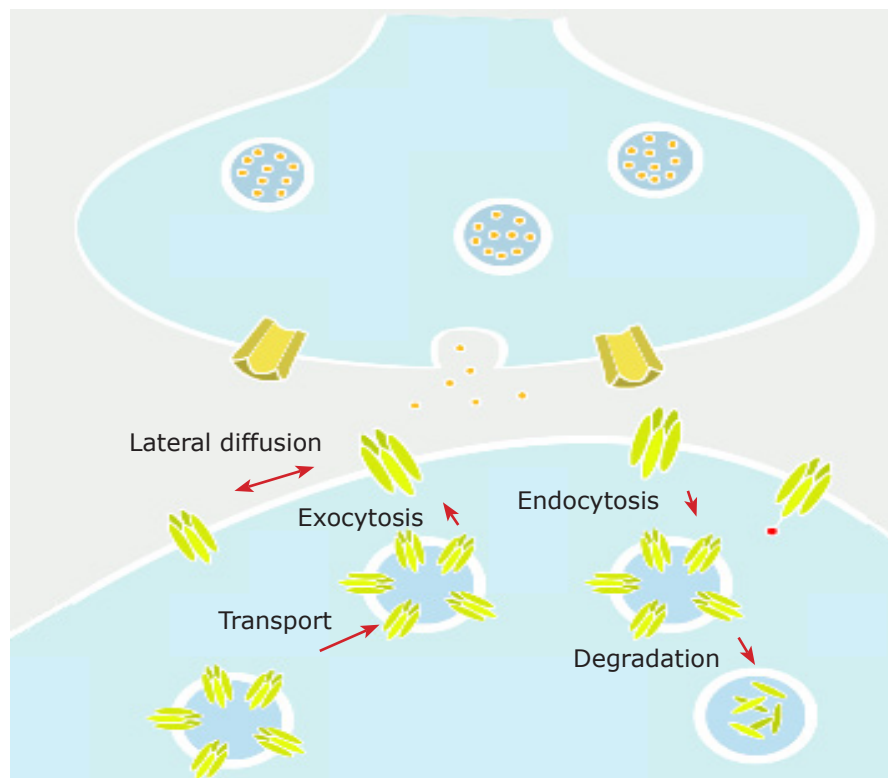
It was recently shown that learning drives AMPA-type glutamate receptors into the synapse of postsynaptic neurons (Rumpel et al., 2005; Whitlock et al., 2006); if AMPA receptor synaptic incorporation is blocked, memory will also be reduced (Rumpel et al., 2005). Since learning and memory critically depend on the trafficking of receptors in the membrane, it is important to study the mechanisms that contribute for synaptic incorporation of receptors. The control of receptor diffusion into an out of the synapse is central for determinant of synaptic strength. The increase in synaptic receptors due to lateral diffusion is now thought to result from a complex set of events involving receptor-scaffolding protein unbinding, untethering of receptors from the cytoskeleton following depolymerization or a change in transmembrane adhesion molecules.

Trafficking within the surface membrane has recently emerged as a key step for regulating synaptic responses. Exocytosis, endocytosis and lateral trafficking have been highlighted as a key process in receptor renewal and concentration at synapses, accounting for the construction and plasticity of synapses in the membrane (Fig 1.3). Recent studies indicate that AMPAR endocytosis occurs in endocytic zones positioned

in the vicinity of the PSD (Blanpied et al., 2002; Petralia et al., 2003; Racz et al. 2004; Lu et al., 2007). Receptors are not simply degraded upon internalization, as previously thought (Gardner and Fambrough, 1979). Instead, a significant number of receptors, rather than being metabolized upon internalization, recycle back into the postsynaptic membrane. Interestingly, both endocytic zones and local receptor recycling are required to maintain a mobile pool of receptors at synapses (Petrini et al., 2009).

#### **1.3.4.3 Molecular determinants of lateral diffusion in the cell membrane – lipids, scaffolds and cytoskeleton**

Membrane compartmentalization explains, in part, the heterogeneous diffusion of receptors in the cell surface. The cohesive forces that assemble and maintain different microdomains in the cell membrane include lipid–lipid, protein–protein, and protein–lipid interactions, as well as sub- and supramembrane effectors (cytoskeletal, extracellular



**Figure 1.3 – Receptor exchanges between synaptic, extrasynaptic and intracellular compartments.** The extrasynaptic receptors can access synapses by lateral diffusion. The surface receptors are exchanged with the intracellular pool by insertion and internalization. The intracellular pool also features receptor synthesis, transport, recycling and degradation.

matrix) (Anderson and Jacobson K, 2002; Kusumi et al. 2004; Kwik et al., 2003; Murase et al., 2004; Nicolau et al., 2006; Lenne et al., 2006).

Biological membranes contain hundreds of lipids with different properties and can undergo dynamic changes in composition. The phospholipids form the major lipid part of biological membranes and are composed of two fatty acids plus a phosphate attached to a glycerol. Their hydrophilic head and hydrophobic tail constitute the basis for the formation of self-assembled phospholipid bilayers or micelles in aqueous environments. The great variety of phospholipid molecular species, the differences in their molecular shapes, physical properties, and their asymmetric distribution in the membrane bilayer possibly contribute to the formation of membrane microdomains (Shaikh et al., 2001). Cholesterol is another essential lipidic component of membranes that influences membrane fluidity, membrane protein trafficking, and consequently regulates neurotransmission and receptor trafficking (Allen et al., 2007; Renner et al., 2009). When introduced into lipid bilayers, cholesterol intercalates between the hydrocarbon parts of the other lipids, filling in the flickering spaces between the acyl chains. Because of its rigid planar structure, cholesterol increases the order of the neighboring acyl chains, making the membranes laterally more condensed and more densely packed. Thus, the physicochemical properties of the membrane are altered; in particular, permeability is decreased and mechanical strength and rigidity increased (Needham et al., 1988; Feigenson and Buboltz, 2001). Additionally, cholesterol can also appear concentrated in microdomains or rafts, which are composed by cholesterol and sphingolipids in the exoplasmic leaflet of the bilipid layer and cholesterol and glycerophospholipids in the endoplasmic leaflet. The lipid rafts can measure several tens of nanometers in diameter and behave as "moving platforms" (Simons and Ikonen, 1997; Pralle et al., 2000).

One of the most important properties of lipid rafts is that they can include or exclude proteins to variable extents based on how well they fit within this organized lipid environment (Simons and Ikonen, 1997;). Proteins with raft affinity include glycosylphosphatidylinositol-anchored proteins, doubly acylated proteins, such as Src-family kinases or the  $\alpha$ -subunits of heterotrimeric G proteins, cholesterol-linked and palmitoylated proteins, and transmembrane proteins, particularly palmitoylated ones (Levental et al., 2010). Thus, lipid rafts serve as platforms that organize different



signaling components into dynamic modules and control their subcellular sorting and efficient function.

Cholesterol plays a crucial role in the generation of ordered domains in the plasma membrane that laterally segregate certain proteins, thus reducing their rate of lateral diffusion and, by virtue of this, increasing clustering and consequently signaling strength (Edidin, 2003; Hancock and Parton, 2005; and Hancock, 2006; Lingwood and Simons, 2010). For these reason, lipid rafts determine the functional properties of membrane-resident proteins like ion channels and transmitter receptors (Allen et al., 2007).

A vast number of components have been identified linking directly or indirectly AMPA and NMDA receptors in the PSD (Newpher and Ehlers, 2007). The number of scaffold proteins in the PSD exceeds the number of receptors by a big margin, ensuring plenty of 'slots' for the various binding partners. The members of the membrane associated guanylate kinases (MAGUKs) family are the most abundant scaffolds in excitatory synapses and are the best-described scaffolds of glutamate receptors (Elias and Nicoll, 2007). This family includes PSD95, PSD93, SAP102 and SAP97. A common feature of all these proteins is that they share the same organization with three N-terminal *post synaptic density protein (PSD95)/Drosophila disc large tumor suppressor (DlgA)/zonula occludens-1 protein (zo-1)* (PDZ) domains, a Src-homology 3 (SH3) domain and a C-terminal catalytically inactive guanylate kinase (GK) domain (Cho et al., 1992; Feng and Zhang, 2009). These domains interact with a relatively weak binding affinity to the small peptide fragments situated at the very carboxyl tail of the scaffolds' targets, thereby ensuring the dynamic range of synaptic responses. Despite the similarities in domain structure, PSD95-like MAGUKs (PSD-MAGUKs) are distinct in their N-terminal amino acid sequences, which could account for the selectivity of their interactions. For example, PSD-95 itself binds directly to the intracellular C-terminal of NMDA receptors (Kornau et al., 1995), and together with other associated PSD-95 molecules, links numerous components in an elaborate postsynaptic scaffold. On the other hand, PSD95 requires a TARP link to bind AMPARs (Hashimoto et al., 1999; Chen et al., 2000), as well to other components important for signal transduction such as calcium/calmodulin-dependent protein kinase II (CaMKII). SAP102 and PSD-93 are related members of the PSD-95 family and perform similar functions at glutamatergic

synapses depending on the developmental stage and location of the synapse (Elias et al., 2006). The fourth member of the family, SAP97, plays a different role, facilitating AMPA receptor trafficking to the surface membrane, for example (Nakagawa et al., 2004).

The cytoskeleton provides the stability for receptor anchoring and controls the “apparent viscosity” of mammalian plasma membranes (Gulley and Reese, 1981; Kusumi et al., 2005). A well-accepted hypothesis is that the cytoskeleton cortex hinders protein movements by controlling (1) the avidity of the postsynaptic scaffold for receptors; (2) creating fences below the membrane or by anchoring transmembrane molecules, which then act as obstacles to lateral diffusion (Kusumi and Sako, 1996; Saxton and Jacobson, 1997) and likely controlling (3) adhesion molecules, which create permeable barriers at the edge of excitatory and inhibitory synapses (Triller and Choquet, 2003). The cytoskeleton actions above described depend on both actin (Juliano, 2002; Yamagata et al., 2003; Bamji, 2005) and microtubules (Barth et al., 1997; Schoenwaelder and Burridge, 1999). Filamentous actin (F-actin) is the main cytoskeletal component and forms a large cortical meshwork that is concentrated beneath excitatory and inhibitory postsynaptic membranes, just below the postsynaptic scaffold (Dillon and Goda, 2005). Microfilaments are important for maintaining synaptic integrity and function and their depolymerization can disrupt the signaling of activated neurotransmitter receptors (Charrier et al., 2006). The cytoskeleton, together with the subsynaptic proteins, might constitute submembranous diffusion fences; adhesion proteins are probably passive obstacles that hinder diffusion, whereas scaffolding molecules probably restrict diffusion through specific interactions with given receptors (Choquet and Triller, 2003).

#### **1.3.4.4 Trafficking of neurotransmitter receptors that underlie synaptic plasticity – the example of long-term potentiation in CA1 hippocampal region**

The most extensively studied and therefore prototypic form of synaptic plasticity is LTP observed in the CA1 region of the hippocampus. In LTP, activation of AMPARs by presynaptically release glutamate must depolarize the membrane to an extension that allows relieving the voltage-dependent block of the NMDAR by magnesium ( $Mg^{2+}$ )

(Coan and Collingridge, 1985). Under these conditions,  $\text{Ca}^{2+}$  enters postsynaptic dendritic spines, activating complex intracellular mechanisms and signaling cascades that include several protein kinases, most notably CaMKII (Ouyang et al., 1999). In a simplified scheme, the levels of  $\text{Ca}^{2+}$  in the postsynaptic neuron determines the nature of LTM, since different cytoplasmic  $\text{Ca}^{2+}$  concentrations recruit different subsets of  $\text{Ca}^{2+}$ -dependent intracellular signaling molecules. For instance, long-term depression (LTD) requires a smaller rise in postsynaptic  $\text{Ca}^{2+}$  than LTP (Malenka and Bear, 2004). In the case of LTP, the primary mechanism underlying the increase in synaptic strength during LTP is a change in AMPAR trafficking that results in an increased number of AMPARs in the postsynaptic plasma membrane with no effect on NMDARs (following the same simple scheme, LTP involves increased exocytosis of AMPARs, whereas long-term depression LTD involves increased endocytosis of AMPARs). The majority of AMPARs incorporated into synapses during LTP is from lateral diffusion of spine surface receptors containing GluR1. Following synaptic potentiation, GluR1-containing AMPARs from intracellular pools are driven to the surface primarily on dendrites (Park et al., 2004). These exocytosed receptors likely serve to replenish the local extrasynaptic pool available for subsequent bouts of plasticity (Makino et al., 2009). Within a few hours, the maintenance of LTM requires protein synthesis.

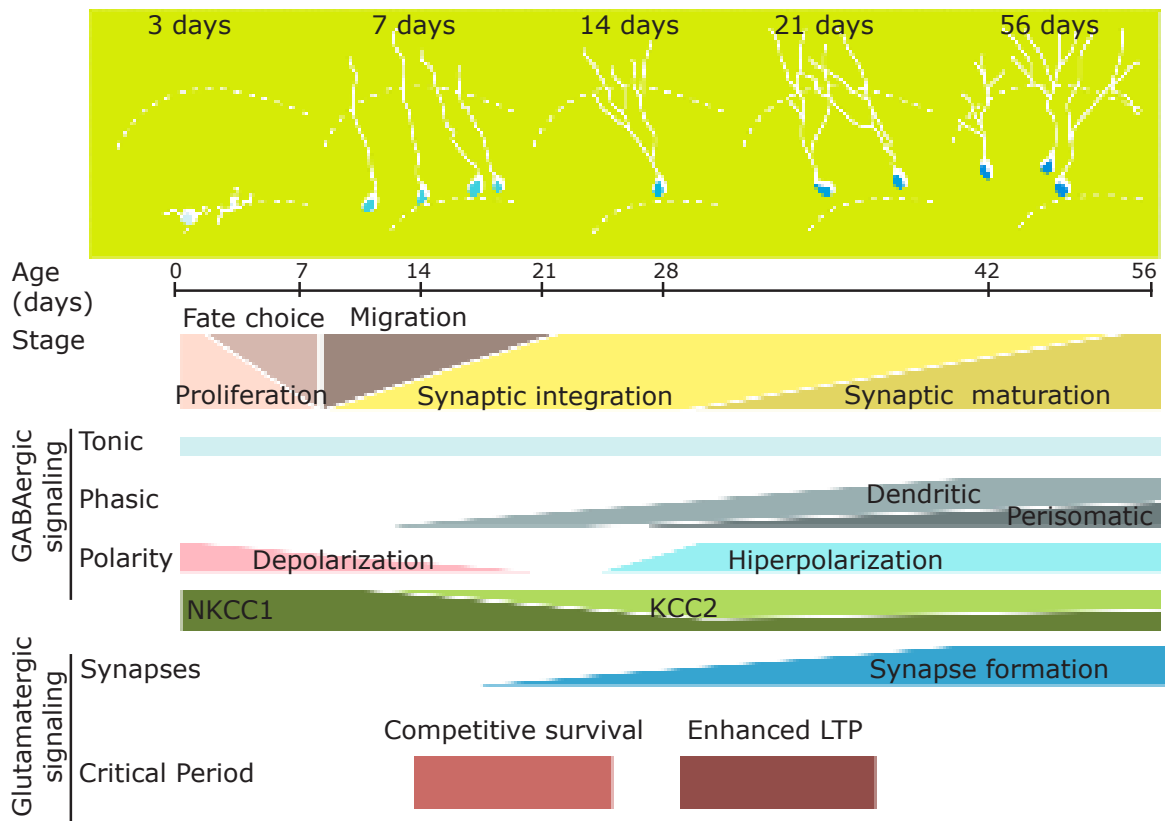
### **1.3.5 New neurons in old brains – adult neurogenesis in the hippocampus**

The plasticity of the brain is ultimately exemplified by its ability to generate new neurons throughout life. The hippocampus is one of the two regions of the adult brain that retains the ability of generating new neurons from stem cells (Altman and Das, 1965; Caviness, 1973; Erikson et al., 1998; Kornack and Rakic, 1999). The function of adult neurogenesis in the hippocampus has been correlated with learning and memory (Shors et al., 2001, 2002; Rola et al., 2004; Snyder et al., 2005; Winocur et al., 2006; Aimone et al., 2009; Clelland et al., 2009; Deng et al., 2009; Deng et al., 2010).

The primary source of neuronal progenitor cells (NPCs) of the DG is the subgranular zone (SGZ), which is located between the inner third of the granule cell layer (GCL) and the *hilus* (Altman and Bayer, 1990). Together with the NPCs of the lateral ventricular

wall, the SGZ is a region of the brain containing NPCs, which are self-renewing, multipotent cells able to generate neurons, astrocytes, and oligodendrocytes (Cameron et al., 1993). This ability is preserved even in the adult brain, and therefore NPCs underlie the phenomenon of adult neurogenesis. It has been estimated that several thousands of new cells are generated every day, but only about 50% of them will survive and finally integrate into neuronal circuits as granule cells (Dayer et al., 2003). Accordingly, neurogenesis includes not only cell proliferation, but also cell survival, neuronal cell fate determination (differentiation) and correct incorporation in the neural network. In the last years, several studies demonstrated that adult-born neurons exhibit unique properties during their maturation stages (Espósito et al., 2005; Overstreet Wadiche et al., 2005; Ge et al., 2006). Therefore, a strategic integration of adult-born neurons into the existing circuitry may be the functional basis of their specific contribution to brain functions. Each developmental stage has its distinct physiological and morphological properties and, to some extent, adult neurogenesis recapitulates embryonic neurogenesis (Espósito et al., 2005). Interestingly, recent evidence arises that due to their unique characteristics, these adult born granule cells may contribute to hippocampal function even before they achieve complete maturation stage (Deng et al., 2009).

During the first week after birth, the adult-born neurons undergo their initial differentiation and migrate a short distance into the inner GCL of the DG (Fig. 1.4), where they extend limited cellular processes, but do not seem to be synaptically integrated in the network. Notably, these cells are tonically activated by ambient GABA (Espósito et al., 2005; Ge et al., 2006). Adult-born DG cells become more neuron-like cells during the second week after birth: they grow polarized processes, with dendrites extending towards the molecular layer and axons growing through the hilus to CA3 (Hastings et al., 2002; Zhao et al., 2006). Nevertheless, these immature neurons are still considerably different from mature dentate granule cells. For example, at this stage, the adult-born neurons lack glutamatergic input (Espósito et al., 2005; Zhao et al., 2006). They receive, however, synaptic GABAergic input, presumably from local interneurons. GABA-mediated responses are very different in a two-week old neuron than in mature granule cells though (Espósito et al., 2005; Overstreet Wadiche et al., 2005; Ge et al., 2006). The spontaneous and evoked GABAergic events show slower



**Figure 1.4 – Adult hippocampal neurogenesis.** The proliferation of neural progenitor cells morphologies gives rise to adult-born dentate granule cells. The fate-committed, adult-born DGCs undergo several stages of development, with gradual changes in morphological and physiological characteristics. About 7 days after birth, the adult-born DGC extends its dendrite into the granule cell layer and molecular layer and projects the axon into the hilus toward CA3. The adult-born DGC receives excitatory GABAergic input, presumably from local interneurons. The GABA is still depolarizing due to a reversed chloride gradient, which is generated by the highly expressed chloride transporter NKCC1. During the third week after birth, the DGC receives glutamatergic input from the perforant pathway. At this stage, the GABA input changes from being excitatory to being inhibitory due to the increased expression of KCC2 (and decreases expression of NKCC1). Both efferent and afferent synapses of the adult-born DGCs begin to form around this time. During the maturation stages, there are two critical periods when new neurons are particularly sensitive to glutamatergic signaling. At around 2 months of age, the basic structural and physiological properties of the adult-born DGCs are indistinguishable from those of mature DGCs. Adapted from Ge et al., 2008.

rise and decay times in immature neurons, probably due to the expression of different receptor subunits (Overstreet Wadiche et al., 2005; Markwardt et al., 2009). Moreover, the nature of the GABAergic response is different. In immature neurons, GABAergic input results in neuronal depolarization due to the efflux of  $\text{Cl}^-$  ions via  $\text{GABA}_A$  channels (Ben Ari et al., 1989). Importantly, the initial period of depolarizing GABA is necessary both for early postnatal and adult-born neurons to develop properly and integrate

into circuits (Rivera, et al., 1999; Ben-Ari 2002; Payne et al., 2003; Ge et al., 2006). The transition of GABAergic input from depolarizing to hyperpolarizing coincides with the onset of glutamatergic inputs and with the timing of synaptic integration. By approximately day 16, spines begin to appear on the dendrites of adult-born neurons, forming synapses with the afferent axon fibres in the Perforant Pathway that come from the EC (Zhao et al., 2006; Toni et al., 2008). Initially, filopodia are frequently present on dendrites (Toni et al., 2007). The majority of the filopodia on the adult-born neurons target axon boutons that already synapse with existing spines on other neurons by forming multiple synaptic boutons. During the third week after birth, adult-born neurons start to form afferent and efferent connections with the local neuronal network, followed by the formation of perisomatic GABAergic inputs (Zhao et al., 2006). For glutamate signaling, glutamatergic synapse formation starts after initial synaptogenesis of GABAergic dendritic synaptic inputs and before synaptogenesis of perisomatic GABAergic synaptic inputs. A prolonged structural modification in dendritic spines takes place then, and the neuron can take several months before reaching total maturation (Zhao et al., 2006).

One hallmark of adult neurogenesis is its regulation by the activity of the existing neuronal circuitry. Immature neurons start to express ionotropic receptors long before they are targeted by synaptic afferents, allowing them to sense transmitters in the ambience. Accordingly, neurotransmitter-mediated receptor activation appears to provide a mechanism through which hippocampal network activity regulates neurogenesis to recruit and integrate the precise number of new neurons for activity-dependent modification of the hippocampal circuitry (Ge et al., 2006; Tashiro et al., 2007; Aimone et al., 2009).

Pioneering work on adult neurogenesis indicates that GABA and glutamate, the main inhibitory and excitatory neurotransmitters, respectively, for mature neurons in the adult brain, play important roles in the development of adult-born neurons. These neurotransmitters activate neurons not only locally within synaptic clefts (phasic activation), but also at a distance after diffusion out of synapses (tonic activation). Ambient GABA starts to tonically depolarize these cells in the first week after birthday (Espósito et al., 2005; Overstreet Wadiche et al., 2005; Ge et al., 2006). It constitutes

the bulk of GABA-induced activation during the initial integration process when the phasic GABA activation either does not exist or is weaker than the tonic activation. The mechanism by which tonic GABA activation regulates neuronal development and synaptic integration of new DGCs in the adult brain remains to be determined.

Glutamatergic activity through NMDA receptors encourages survival of adult-born neurons during a critical period when the neurons are first assimilated into behaviorally relevant networks (Tashiro et al., 2006; Tashiro et al., 2007). During the maturation stages, there are two critical periods when new neurons are particularly sensitive to glutamatergic signaling, the first involving NR1-dependent competitive survival of new neurons and the second involving NR2B-dependent enhanced synaptic plasticity (Fig. 1.4).

In addition, in the adult hippocampus there are more neurotransmitters other than glutamate and GABA that could be important for adult neurogenesis as well. Recently, it has been suggested that monoamines and acetylcholine also play a role in the development of adult-born neurons (Brezun and Daszuta, 1999, 2000; Kulkarni et al., 2002; Cooper-Kuhn et al., 2004; Kaneko et al., 2006). The receptors and mechanisms by which they exert their effects, as well as possible interactions with other classes of neurotransmitters and/or growth factors remain to be determined.

#### **1.4 NICOTINIC ACETYLCHOLINE RECEPTORS**

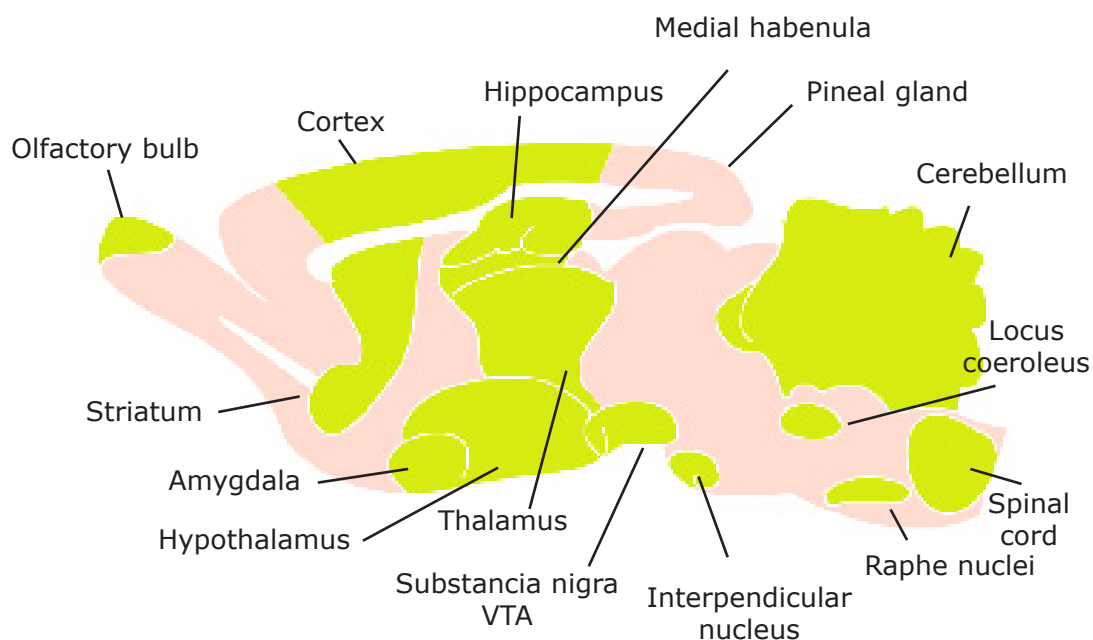
nAChRs belong to the superfamily of ionotropic ligand-gated ion channels that includes the serotonin 5-HT<sub>3</sub>, GABA<sub>A</sub>, and Gly receptor channels (Lester et al., 2004; Sine and Engel, 2006), and can be activated by the agonists acetylcholine and nicotine. nAChRs are cationic-permeable channels, and their ongoing activity directly or indirectly contributes to Ca<sup>2+</sup> signals that regulate several intracellular pathways (Vernino et al., 1992; Séguela et al., 1993; Fucile, 2004).

Neuronal nAChRs are widely expressed in the peripheral and central nervous system where they are involved in a variety of physiological processes (Fig. 1.5). Fast, direct nicotinic synaptic transmission drives NMJ and autonomic ganglion synaptic transmission (Albuquerque et al., 2009). Only rare cases of fast nicotinic transmission have been reported in the mammalian brain, namely in the hippocampus, in the

supraoptic chiasmatic nucleus, and in the cortex (Roerig et al., 1997; Alkondon et al., 1998; Frazier et al., 1998; Hefft et al., 1999; Albuquerque et al., 2009). For this reason, neuronal nAChRs in the brain are more commonly associated with modulatory events rather than mediation of synaptic transmission (McGehee et al., 1995; Gray et al., 1996; Aramakis and Metharate, 1998; Alkondon and Albuquerque, 2001; Ji et al., 2001; Le Magueresse et al., 2006; Zhang and Berg, 2007).

By subtly influencing various aspects of neuronal communication, nicotinic mechanisms contribute to the overall efficiency of circuits and affect information processing and storage (Picciotto et al., 1995; Jones et al., 1999; Bannon et al., 1998; Maskos et al., 2005; Bitner et al., 2007). Because cholinergic neurons are usually loosely distributed in the brain and sparsely innervate broad areas, it has been experimentally difficult to stimulate a large number of cholinergic neurons and to record from the precise location of their innervation. It is likely, however, that fast nicotinic transmission is present at low densities in more neuronal areas than the few that have been reported so far.

Evidence from a multitude of studies converges to the conclusion that nAChRs are located at least at one of five primary locations: the cell soma, dendrites, preterminal axon regions, axon terminals, and myelinated axons on the neurons (Fabian-Fine et



**Figure 1.5 - Distribution of nAChRs in the brain.** Regions expressing nAChRs are shown in green.



al., 2001; Grybko et al., 2010). More recently, it was shown that nAChRs are also expressed in glia cells, including astrocytes (Sharma and Vijayaraghavan, 2001). Due to the broad sub-cellular distribution of nAChRs, they are in a position to regulate the strength of synapses at different levels. Modulation of neurotransmitter release by presynaptic nAChRs is the most prevalent and well-studied nicotinic role in the CNS. Direct or indirect  $\text{Ca}^{2+}$  influx through preterminal nAChRs promotes the release of several neurotransmitters, including glutamate and GABA (McGehee et al., 1995; Alkondon and Albuquerque, 2001; Maggi et al., 2001; Le Magueresse et al., 2006). Most of nAChRs have a high permeability to  $\text{Ca}^{2+}:\text{Na}^+$ , which usually falls within a ratio 3-4:1 (Fucile, 2004). In the case of  $\alpha 7$ -nAChR, its relative permeability of  $\text{Ca}^{2+}:\text{Na}^+$  ratio is  $\geq 10$ , exceeding that of the glutamate NMDA receptor (Bertrand et al., 1993; Séguela et al., 1993). Since nAChRs constitute ionic channels by themselves, their activation also alters membrane resistance and, consequently, changes the space constant of the cellular membrane. These intrinsic properties of neurons influence the spread and efficiency that a synaptic input to produce an AP output in the target cell. In addition, axonal, dendritic and somatic nAChRs also modulate transmitter release and local excitability (Alkondon et al., 2009).

#### **1.4.1 Nicotinic acetylcholine receptors: subunits and subtypes in the nervous system**

nACRs can be made up from a portfolio of different subunits. nAChRs are either homo- or heteromeric assemblies of five subunits, with each subunit arranged around a central pore (for review see Corringer et al., 2000; Unwin, 2005; Sine and Engel, 2006). The  $\alpha$  subunits carry the principal components for the agonist/competitive antagonist binding sites and the  $\beta$  (or non- $\alpha$ ) subunits bear the complementary component (Arias, 2000). At present, 17 nicotinic subunits have been cloned, the muscle  $\alpha 1$ ,  $\beta 1$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits, and the neuronal  $\alpha 2 - \alpha 10$  and  $\beta 2 - \beta 4$  subunits (Albuquerque et al., 2009). Muscle nAChRs are a clear example of the strict association between subunit composition and function. nAChRs are hemopentameric channels that, in the case of the muscle subtype, can be constituted by a set of five subunits -  $\alpha 1$ ,  $\beta 1$ ,  $\gamma$ ,  $\delta$  or  $\epsilon$ . Under these conditions and from a pure mathematical point of view, cells could assemble 126 different

subtypes of nAChR. However, in nature, muscle cells only express two different nAChRs combinations, pointing out the existence of mechanisms that tightly ensure a proper stoichiometry of functional nAChRs. Early in development, when muscle cells are not innervated, nAChRs are found in a  $(\alpha 1)_2\beta 1\gamma\delta$  combination, which confers a long-lasting open channel time and leads to an intermittent depolarization that favors receptor clustering (Mishina et al., 1986; Gu and Hall, 1988). Interestingly, this depolarization also leads to the expression of the  $\epsilon$  subunit (Goldman et al., 1988), which competes the  $\gamma$  subunit for assembly in the receptor. The  $(\alpha 1)_2\beta 1\epsilon\delta$  combination, which can be found in the adult (or innervated) muscle cell, is more stable to degradation, aggregate at the neuromuscular junction with a higher density and exhibit a faster response to the agonist (Gu and Hall, 1988; Missias et al., 1996).

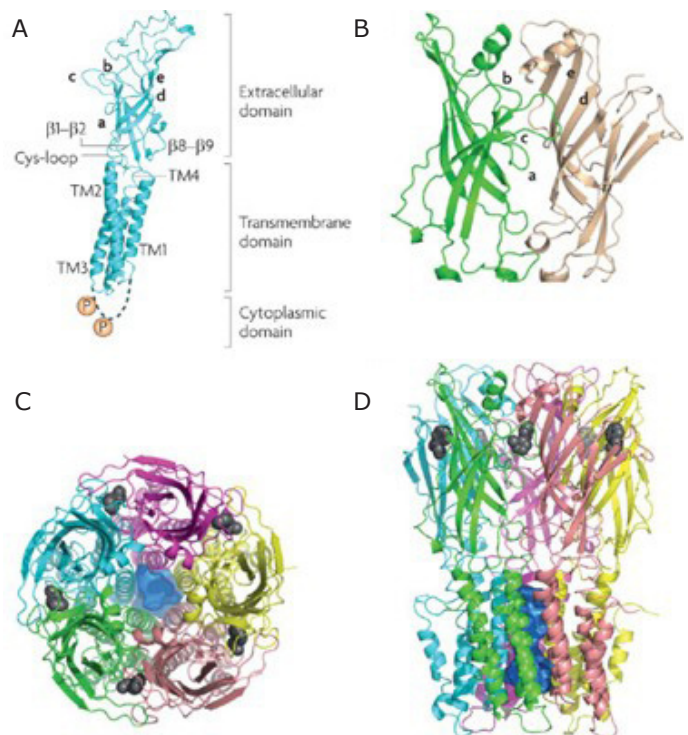
In the case of neuronal nAChRs, the number of different subunits found in nature is even higher than for muscle nAChRs. To date, 9  $\alpha$ -like subunits and 3 non- $\alpha$  subunits have been clone from mammalian neuronal tissue (Corringer et al., 2009). Neuronal subunits that form nAChRs in  $\alpha\beta$  combinations include  $\alpha 2$ – $\alpha 6$  and  $\beta 2$ – $\beta 4$ . Subunits capable of forming homomeric nAChRs are  $\alpha 7$ – $\alpha 9$ , and  $\alpha 10$  forms a heteromer with  $\alpha 9$  (Dani and Bertrand, 2007). The  $\alpha 8$  subunit has been found in avian tissue but has not been detected in mammals (Dani and Bertrand, 2007). Although there are many potential combinations of neuronal nAChRs, only a few have been found to be of biological importance.

nAChRs can be assembled with different stoichiometries, which can impact on the receptor function and upregulation. For example, the mammalian high-affinity nicotine binding receptor consists of at least  $\alpha 4$  and  $\beta 2$  nAChR subunits (Albuquerque et al., 2009).  $\alpha 4\beta 2$ -containing receptors can be constructed to the final stoichiometry of  $(\alpha 4)_2(\beta 2)_3$  and  $(\alpha 4)_3(\beta 2)_2$  [and sometimes  $(\alpha 4)_2(\beta 2)_2(\alpha 5)$ ] (Nelson et al., 2003; Zhou et al., 2002). While all these three forms of nAChRs bind nicotine with high affinity, it is the  $(\alpha 4)_2(\beta 2)_3$  nAChR that is most sensitive to the upregulation by nicotine (López-Hernández et al., 2004). The high diversity of subunits and the possibility of different stoichiometries contribute, in part, to the multiplicity of roles played by nAChRs in physiological and pathological conditions.

### 1.4.2 Structure of nAChR subunits – Implications for function

Each nAChR subunit has a structure consisting of a conserved and large extracellular N-terminal domain, four hydrophobic transmembrane (TM) domains (TM1-TM4), a short cytoplasmic loop between TM1-TM2, a large cytoplasmic loop between the TM3-TM4, and a short extracellular C-terminus (Fig 1.6; Unwin, 2005; Albuquerque et al., 2009). Common to all subunits is the occurrence of a cysteine loop (Cys loop) in the first portion of extracellular N-terminal domain (Lester et al., 2004).  $\alpha$  subunits have two adjacent cysteines, in addition, near the entrance of TM1 that seem to be essential for ACh binding. The nicotinic binding site lies at the interface between an  $\alpha$ -subunit (the “principal” component, or positive face) and a non- $\alpha$  subunit (the “complementary” component, or negative face), except in homomeric nAChRs (Brejc et al., 2001). The hydrophobic residues determine the ligand binding affinity, whereas the residues of the negative face determine ligand selectivity (Brejc et al., 2001). The extracellular N-terminal domain also serves as the major binding site for antagonists like  $\alpha$ -bungarotoxin ( $\alpha$ -Bgt) and other snake toxins, which are potent inhibitors of

**Figure 1.6 - Model of the  $\alpha 7$ -nAChR.** Comparative modelling based on the homologue protein from *Erwinia chrysanthemi* (Protein Data Bank code 2VL0). A) Structure of one subunit of the  $\alpha 7$ -nAChR model. The cytoplasmic domain and its phosphorylation sites are schematically represented, as they are not present in the model. The ‘loops’ of the binding site (a–e) are labelled as well as the loops of the interface between the extracellular and transmembrane (TM) domains (cys-loop,  $\beta 1$ – $\beta 2$  and  $\beta 8$ – $\beta 9$ ). B) Close view of the acetylcholine-binding site. For clarity, only two monomers are represented. The loops of the binding site (a–e) are labelled. C,D) Top view (C) and side view (D) of the  $\alpha 7$  nAChR pentamer, showing five nicotine molecules (dark grey) in the binding sites and the volume of the ion channel (dark blue). Adapted from Taly et al., 2009.



nAChRs.

The four TM domains are  $\alpha$  helices packed around the central hydrophilic pore. The TM2 lines the pore, while TM1, TM3 and TM4 segments separate the pore-lining region from the hydrophobic membrane (Karlin, 2002; Albuquerque et al., 2009). TM4 is away from the pore and mostly interactive with the lipid bilayer. TM1 and TM3 complete this helix bundle by positioning opposite to each other and rotated by 90° relative to TM2 and TM4 (Unwin et al., 1998; Unwin et al., 2005).

TM1 has a minor contribution in the pore structure the nAChR channel, only lining the region where the pore widens toward the extracellular membrane surface. The pore is mainly constituted by the transmembrane domain TM2 and is critical for establishing the ion gate, cation-selectivity, and channel conductivity (Dani et al., 2007; Albuquerque et al., 2009). Ligand binding induces rotation of the extracellular domain and this is translated into rotation of the TM2 helices. This step has three different consequences: 1) removal of hydrophobic barrier residues from the pore, 2) an increase in the pore diameter and 3) movement of hydrophilic residues into the channel to support ion flow (Gao et al., 2005). Computational simulations predicted that the greatest structural change during gating of nAChR occurs in TM4. This might be due to the exquisite location of TM4 in the cell membrane, where it establishes relative few contacts with proteins in comparison with other TMs. In fact, TM4 contains a highly conserved cysteine residue that appears to be involved in receptor aggregation and interaction with cholesterol and other lipid-related molecules such as sterols (Barrantes, 2004). Consequently, manipulation of the lipid content or the degree of receptor aggregation has potential to modify the gating mechanism and may even have some functional consequence. TM1 and TM3 also seem to interact cholesterol (Taly et al., 2009), although in a less extension than what is described for TM4.

The amino acid sequence of the large cytoplasmic loop between TM3 and TM4 is unique among eukaryotes and shows considerable diversity among different nAChR subunits, suggesting that this region might be important for subunit-specific behavior and interactions with cellular components. To date, it seems that most of the cytoplasmic loop is unordered, which is actually important for the function of some proteins, and could eventually explain the various functions of this region in the resting, open and

desensitized states of nAChR (Kukhtina et al., 2006). The large cytoplasmic loop is thought to interact with proteins involved in trafficking and targeting, scaffolding, as well as with kinases and other signaling proteins, making this domain important when considering assembly, expression and function of nAChRs (Kuo et al., 2005). The large cytoplasmic loop also harbors sequences important to the distribution of receptors on the cell surface. For example, it was shown that the cytoplasmic loop of  $\alpha 3$  subunit targets  $\alpha 3^*$ -nAChRs at the synapses, in ciliary ganglion in vivo (Williams et al., 1998). In contrast, sequences within the  $\alpha 7$ -nAChR subunits exclude  $\alpha 7$ -nAChRs from the synapse and favor their perisynaptic localization (Williams et al., 1998).

### **1.4.3 Posttranslational modifications of nAChRs**

Receptor expression seems to be regulated by a combination of intrinsic structural features of the respective receptor and the ability of the cell to recognize and modify the structural sequence in a manner favorable to subsequent receptor expression at the surface. Multiple sites of nAChRs subunits important for receptor folding, assembly and trafficking can be glycosylated, phosphorylated and palmitoylated (Alkondon et al., 2009). Consequently, posttranslational modification control at some extent the subcellular localization and function of nAChRs in the cell surface. Glycosylation, in which a carbohydrate chain is enzymatically attached to a protein, seems to be important for the surface expression of the receptor and to the ability of nAChR channels to open in response to the agonists (Gehle et al., 1997; Dellisanti et al., 2007). Phosphorylation within the cytoplasmic domain occurs for several nAChRs subtypes; the functional consequence of this posttranslational modification is specific for each receptor subtype, location and cell type, even when despoiled by the same signaling pathway (Wiesner and Fuhrer, 2006). Palmitoylation is another posttranslational and reversible process that takes place in the ER, where palmitate is covalently attached to cysteine residues via thioester bonds that serves to anchor the proteins to either face of the cell membrane (Linder and Deschenes 2004; Smotrys and Linder 2004; Resh 2006). Palmitoylation occurs for many of the muscle and neuronal nAChRs subunits and, therefore, is likely to have a role in the assembly, trafficking and/or function of nAChRs (Alexander et al., 2010a). It is believed that the palmitate inserts and assumes a place in the bilayer

structure of the membrane alongside the similar fatty-acid tails of the surrounding lipid molecules. There is some evidence however that the palmitate does not act merely as an anchor. It is possible that these additional lipid tails help the proteins to be properly sorted in the cell membrane, targeting them to specific domains, such as lipid rafts, or altering their conformation to regulate their activity and interaction with other proteins (Allen et al., 2007).

#### **1.4.4 Trafficking of nAChRs – putting nAChRs in the cell surface**

##### **1.4.4.1 Chaperones and scaffolds as intrinsic factors that regulate the trafficking of nAChRs**

The broad spectrum of functions and locations of nAChRs at pre- and postsynaptic places throughout the nervous system raises the issue of how the cellular distribution of these receptors is regulated. nAChRs are likely to be localized in defined cell compartments based on a combination of subunit composition and the presence of scaffold components and associated transmembrane proteins that localize them in their final destination (Conroy et al., 2003, 2007; Parker et al., 2004; Temburni et al., 2004; Farias et al., 2007; Rosenberg et al., 2008). The specific sites for insertion/endocytosis of neuronal nAChRs and the mechanisms that regulate their lateral diffusion on the cell membrane are not known yet. Trafficking neuronal nAChR seems to be distinct from the muscle subtype, since different proteins/scaffolds associate with them (Millar and Harkness, 2008). For instance, rapsyn was one of the first nAChR interactors to be identified, and it was found to promote clustering of nAChRs at the postsynapse of the NMJ (Froehner et al., 1990; Gautam et al., 1995). As in muscle, neuronal nAChRs are clustered, but rapsyn, which has been detected in the nervous system, is incapable of clustering  $\alpha 3\beta 2$  or  $\alpha 4\beta 2$  nAChRs at the cell surface (Huh and Fuhrer, 2002). In addition, it was observed that the clustering of  $\alpha 5$ - and  $\beta 2$ -containing nAChRs is unaffected in mice lacking rapsyn, indicating that rapsyn is not necessary for neuronal nAChR clustering (Feng et al., 1998).

Recent proteomic studies of purified mice brains identified at least 21 proteins

associated with the  $\beta 2$  subunit and 57 with the  $\alpha 7$ -nAChR (Kabbani et al., 2007; Paulo et al., 2009); several of these proteins are implicated in regulation of sub-cellular trafficking. Pursuing the functional meaning of these interactions in the future will help to clarify the machinery involved in the trafficking of nAChRs to, from and within the cell membrane.

Once synthesized, neuronal nAChR associate with chaperone proteins that transport receptors away from the ER. These chaperones associate with the precursors of nAChR subunits to enhance and favor subunits' folding into complete complexes. The dependence on chaperones and scaffolds to promote the assembly and trafficking of nAChRs to the cell membrane may be most pronounced for  $\alpha 7$ -nAChRs; RIC-3 has been identified as a chaperone indispensable for assembly and trafficking of  $\alpha 7$ -nAChRs to the cell surface (Halevi et al., 2002, 2003; Ben-Ami et al., 2005; Castillo et al., 2005; Lansdell et al., 2005; Williams et al., 2005; Wang et al., 2009). More recently, it was shown a dual role for RIC-3 on the expression of  $\alpha 7$ -nAChRs (Alexander et al., 2010b). When present at low levels, RIC-3 interactions are short-lived and promote  $\alpha 7$ -nAChRs assembly and release from the ER. At higher levels, RIC-3 interactions are longer-lived and mediate the retention of  $\alpha 7$ -nAChRs at the ER (Alexander et al., 2010b). Receptor internalization is also likely to depend on specific scaffold components and contribute importantly to the regulation of nicotinic signaling. One example is provided by the SNARE-dependent activity-induced internalization of  $\alpha 7$ -nAChRs (Liu et al., 2005).

Some of the proteins that contribute for the clustering of nAChRs on the cell membrane were already identified. The tumor-suppressor protein adenomatous polyposis coli (APC) targets  $\alpha 3^*$ - but not  $\alpha 7$ -nAChRs to postsynaptic sites in chick ciliary ganglion (CG) neurons (Temburni et al., 2004; Rosenberg et al., 2008; Rosenberg et al., 2010). Interestingly, presynaptic  $\alpha 7$ -nAChRs co-localized with the scaffold APC in hippocampal neurons (Farías et al. 2007), pointing out the cell-specificity of receptor-scaffold interactions.

APC's binding partners in CG neurons are the End binding protein 1 (EB1), postsynaptic density protein PSD-93, and  $\beta$ -catenin. Simultaneous block of APC's interactions with both EB1 and PSD-93 caused specific decreases in  $\alpha 3^*$ -nAChR clusters (Temburni et al., 2004). APC directs essential aspects of synaptic assembly by organizing a complex of



EB1, key cytoskeletal regulators macrophin and IQGAP1, and 14-3-3 adapter proteins at postsynaptic sites. The same authors showed that expression PSD-93, *in vivo*, caused no significant change in  $\alpha 3^*$ -nAChR surface labeling. However, immunoprecipitation of solubilized components shows that PSD-MAGUK family members, including PSD-93, form a complex with ganglionic nAChRs (Conroy et al, 2003). Subunit specificity in these interactions is suggested by numerous findings. For example, in CG neurons, PSD-95 was shown to associate with  $\alpha 3$ - and  $\alpha 5$ -containing nAChRs, but not  $\alpha 7$ ,  $\alpha 4\beta 2$  or muscle nAChRs (Conroy et al, 2003). To date, specific sequences that facilitate the interaction between nAChRs and PDZ complexes have not been identified yet. It is not clear whether the interactions between nAChRs and PSD-MAGUK family members are direct or indirect in those cases, but it is becoming evident that these scaffold proteins are essential for mediating nAChR function in the autonomic nervous system. Denervation studies demonstrate that PSD-93 promotes synaptic stability in superior cervical ganglion neurons and submandibular ganglion neurons; synaptic clusters of nAChRs disperse much more rapidly in mice lacking PSD-93 (Parker et al., 2004). More recently, it was shown that axotomy leads to the loss of synaptic PSD93 that precedes the loss of nAChRs in submandibular ganglion (McCann et al., 2008). Disruption of PDZ scaffolds also interferes with signal transduction and downstream signaling by nAChRs (Conroy et al., 2003).

Best characterized are the roles of PSD-MAGUK family members in regulating nAChR function on autonomic neurons, but postsynaptic PDZ-scaffolds at nicotinic synapses also occur in the CNS. For instance, in hippocampal neurons,  $\alpha 7$ -nAChRs appear to co-localize with PSD-95 (Xu et al., 2006). Other scaffold protein that controls  $\alpha 7$ -nAChR clustering in hippocampal neurons is PICK1 (Baer et al., 2007).

In order to synapses form and function properly, neurotransmitter receptors must be recruited to locations on the post-synaptic cell in direct apposition to pre-synaptic neurotransmitter release. Once inserted into the postsynaptic membrane, receptors are not fixed in place though; receptors are mobile in the plasma membrane and traffic to sites of nerve-muscle contact or neuron-neuron contact (Newpher and Ehlers, 2008). The first studies demonstrating the mobility of neurotransmitter receptors in the cell surface focused on muscle nAChRs (Anderson and Cohen, 1977; Axelrod et al., 1978; Young and Poo, 1983). That neuronal nAChRs rapidly exchange between synaptic and



extrasynaptic regions was only recently shown (McCann et al., 2008), almost 30 years after the first reports on the NMJ. There are some important differences that should be considered when looking at the dynamics of muscle and neuronal nAChRs. First, these two types of receptors are expressed in different cellular and molecular environments (muscle and neurons), and second they have a different subunit composition. As a consequence, nAChRs interact and/or associate with different molecular partners on muscle cells and neurons. Knowledge on the specific mechanisms that regulate the trafficking of neuronal nAChRs is still very limited.

#### **1.4.5 Signaling cascades mediated by nAChRs**

Although traditionally thought to serve primarily as receptor anchors or placeholders, receptor-associated proteins have been shown to play a central role in coupling neurotransmitter receptors with specific intracellular signaling cascades (Neff et al., 2009a). Once nAChRs are activated, the channels open and allow the influx of cations. Since nAChRs are strong inward rectifying channels, they are active at hyperpolarised or resting membrane potentials and show attenuated inward currents at strongly depolarised potentials (Albuquerque et al., 1995). nAChRs, specially the  $\alpha 7$  subtype, are permeable to  $\text{Ca}^{2+}$  (Bertrand et al., 1993; Seguela et al., 1993). In addition, nAChR activation can indirectly induce an increase in cytoplasmic  $\text{Ca}^{2+}$  through the activation of VGCC and/or from intracellular  $\text{Ca}^{2+}$  stores (Dajas-Bailador and Wonnacott, 2004). For these reasons, nAChR activation has been have been implicated in  $\text{Ca}^{2+}$  entry-dependent events, including neurotransmitter release and regulation of secondary messenger cascades (Dajas-Bailador and Wonnacott, 2004). Both protein kinase A (PKA)- and protein kinase C (PKC)-dependent signalings have been implicated in the regulation of/by nAChRs (Downing and Role, 1987; Nishizaki and Sumikawa, 1998; Klein and Yakel, 2005; Shen et al., 2009). Ultimately, nAChR-mediated signaling can lead to alterations in gene expression, as originally shown for immediate early genes and genes involved in transmitter synthesis (Chalazonitis and Zigmond, 1980; Greenberg et al., 1986; Pelto-Huikko et al., 1995; Salminen et al., 1999; Gueorguiev et al., 2000).

The pathways activated by nAChRs are also involved in the regulation of their own function, supporting the idea that receptors are regulated by components tethered in their immediate vicinity. The inactivation or rundown of  $\alpha 7$ -nAChRs depends on receptor activation, cytoplasmic  $\text{Ca}^{2+}$ , calmodulin function, and CaMKII activity (Liu and Berg, 1999). In the same study, it is also reported that a parallel path using many of the same intermediates (but ending with calcineurin activity instead of CaMKII) opposes this activity-dependent inactivation. The state of the cell and internal milieu determine the outcome by determining the balance of the calcineurin and CaMKII responses (Liu and Berg, 1999).

#### **1.4.6 Function of nAChR in the nervous system**

The diversity of nAChRs and their widespread cellular and subcellular localization suggest that they play different roles in the nervous system. It has been described that cholinergic systems are involved in arousal, sleep-wakefulness, locomotor's behavior, learning and memory (Woolf, 1991; Dani and Bertrand, 2007).

Functional cholinergic synapses have been identified in a number of peripheral neurons, like parasympathetic ganglions and sympathetic ganglions, and in muscle cells. In the peripheral nervous system (PNS), nAChRs often mediate excitatory synaptic transmission (Albuquerque et al., 2009). Postsynaptic structures and functional aspects of interneuronal cholinergic synapses are best characterized in chick ciliary ganglia, where developmental and innervation profiles have been intensively investigated. Chick ciliary ganglion neurons express  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 2$ ,  $\beta 4$ , and  $\alpha 7$  subunits (Conroy and Berg, 1995). In these cells, two major combinations of nAChRs subunits have been identified: the  $\alpha$ -bungarotoxin sensitive component comprised of  $\alpha 7$  subunits and the monoclonal antibody (mAb) 35-immunoreactive nAChRs (which can be found at least in two different classes, depending on the presence of  $\beta 2$  subunits, e.g.,  $\alpha 3\alpha 5\beta 2\beta 4$  and  $\alpha 3\alpha 5\beta 4$ ). Whereas clusters of  $\alpha 3/\alpha 5$  subunit-containing ganglionic receptors are found at PSDs and extrasynaptic sites (Williams et al., 1998),  $\alpha 7$ -containing receptors, which represent the most abundant population of ganglionic receptors, are exclusively localized on perisynaptic somatic spines (Shoop et al., 1999).

Cholinergic neurons were also identified in the CNS. To date, there were identified three major cholinergic subsystems above the brainstem that innervate nearly every neural area. One system sends widespread projections from the pedunculo-pontine tegmentum and the laterodorsal pontine tegmentum to the thalamus and midbrain dopaminergic areas and also to the caudal pons and brain stem (Woolf, 1991). The second cholinergic system arises from various basal forebrain nuclei projecting through the cortex and the hippocampal formation (Woolf, 1991). These two systems provide broad, diffuse, and generally sparse innervation to wide areas of the brain. The third major cholinergic subsystem is an exception to this principle of broad innervation; it consists of striatal cholinergic interneurons, which project throughout the striatum and the olfactory tubercle (Zhou et al., 2002).

Information regarding building up and maintaining interneuronal cholinergic synapses in the CNS is limited, mainly due to the diffuse cholinergic innervation and to the relatively low levels of expression of nAChRs in some brain areas. The majority of neuronal nAChRs in the brain fall into two categories: those that bind agonist with high affinity (nM concentrations), and those that bind with lower affinity ( $\mu$ M concentrations). The low-affinity receptors are presumably homomeric  $\alpha 7$  receptors that are  $\alpha$ -bungarotoxin sensitive, whereas high-affinity nicotinic receptors are  $\alpha 4\beta 2$ -nAChRs, which account for >90% of in the brain (Whiting and Lindstrom, 1986). Their physiological contributions to neurotransmission, signaling, and behavior are not completely understood.

#### **1.4.6.1 Cholinergic signaling in the hippocampus**

The septum/nucleus of the diagonal band of Broca (nDBB) is the primary source of cholinergic input to hippocampus (Lewis et al., 1967; Mellgren and Srebro, 1973; Mosko et al., 1973; Lynch et al. 1977). The cholinergic innervation enters via the fornix/fimbria and diffusively projects to all hippocampal areas, establishing direct synaptic contacts onto pyramidal cells, granule cells, interneurons, and neurons of the hilus. In addition to this directed input, a significant proportion of cholinergic release sites do not associate with postsynaptic specializations, suggesting an additional bulk transmission role (Lendvai and Vizi, 2008).

Cholinergic septohippocampal neurons are believed to produce various rhythms in the hippocampus (Gogolak et al., 1968; McQuiston, 2010), influencing attention and memory processing (Chiba et al., 1995; Hasselmo, 2006). Several lines of morphological data demonstrate that both major classes of cholinergic receptors (muscarinic and nicotinic) are expressed in the hippocampus. In contrast to nAChRs, the muscarinic AChRs (mAChRs) are sensitive to muscarine and constitute G protein coupled receptors that activate phosphoinositide-specific PLC  $\beta$  and  $\text{Ca}^{2+}$  release from internal stores (Eglen, 2006). Due to the characteristics of mAChR signaling, it is considered that they have slower and longer lasting effects than nAChRs.

The hippocampus expresses a wide variety of nAChR subunits, although  $\alpha 7$ ,  $\alpha 4$ , and  $\beta 2$  predominate (Dani and Bertrand, 2007). The diffuse extracellular ACh signal and the broad distribution of nAChRs at synaptic and nonsynaptic locations ensure multiple targets and a variety of nicotinic responses in the hippocampus (Lendvai and Vizi, 2008).

An important aspect of the diffusive nicotinic signal is the fact that the eventual hydrolysis of ACh creates choline (Ch), which activates/desensitizes nAChRs by itself in a subtype-selective manner. Therefore, ACh provides a diffuse, volume signal that continues as a longer-lived Ch signal that acts both at an ongoing background level and at higher concentrations in specific microdomains. Although ACh and Ch activate  $\alpha 7$ -nAChRs with similar single channel open time and conductance, Ch dissociates more rapidly from the receptor and, consequently, induces a less stable state than ACh does (Mike et al., 2000). It is tempting to speculate, however, that during maturation of the nervous system choline acts as the primary endogenous  $\alpha 7$ -nAChR agonist, because expression of the ACh-synthesizing enzyme choline acetylcholine acetyltransferase lags behind the appearance of nAChRs in developing neurons. It is yet to be determined whether any circumstances Ch rather than ACh serves as an endogenous neurotransmitter to activate  $\alpha 7$ -nAChR.

Levels of the  $\alpha 7$ -nAChR are high in the hippocampus, where it can fulfill several roles early on during development. At the anlage of the hippocampal formation, the mRNA and protein for  $\alpha 7$ -nAChR are detected on embryonic day (E) 13 and on E15, respectively, which is long before the entry of cholinergic innervation into that brain

structure (Adams et al., 2002). Several studies report that the septohippocampal pathway appears primarily in the hippocampus only after birth (however, a study found axons of the septohippocampal projection in the hippocampal formation as early as E17) (Linke and Frotscher, 1993; Adams et al., 2002;) In any case, activation of the  $\alpha 7$ -nAChRs before the initial appearance of these early septohippocampal projections would require an endogenous ligand other than acetylcholine.

During the first few weeks of postnatal life, the rodent brain undergoes extraordinary development. In rodents, as well in other mammals, brain development is far from complete at birth, and many neuronal systems mature in response to the continuous interaction with the changing environment. This is especially true for brain areas involved in higher cognitive functions, such as the hippocampus. The expression of  $\alpha 7$ -nAChRs dramatically increases during this period, reaching the highest levels of expression for the entire life span (Tribollet et al., 2003).  $\alpha 7$ -nAChR-mediated actions appear to be important during these times, participating in several  $\text{Ca}^{2+}$ -dependent events that contribute to the maturation of the neuronal network. The activation of  $\alpha 7$ -nAChRs facilitates normal neuronal loss in the ciliary ganglion during development (Hruska and Nishi, 2007). If this action is extensive to areas of the CNS was never investigated. Interestingly, excessive activation of  $\alpha 7$ -nAChR can also culminate in abnormal and massive cell death. For instance, transgenic mice homozygous for a gain-of-function mutation in  $\alpha 7$ -nAChRs exhibit high neuronal apoptosis levels in the cortex and die shortly after birth (Orr-Urtreger et al., 2000).

Besides playing a role in neuronal survival,  $\alpha 7$ -nAChR-mediating signalling was shown to be important for maturation of postnatal neurons. One of the characteristic features of these early postnatal neurons in the hippocampus is their high intracellular  $\text{Cl}^-$  concentration, which is determinant on dictating the nature of GABAergic signaling (Liu et al., 2006). In the immature hippocampus, activation of  $\text{GABA}_A$  receptors leads to the passive efflux of  $\text{Cl}^-$ , resulting in an inward ionic current (since  $\text{Cl}^-$  is negatively charged) able to depolarize cells membrane (Ben-Ari et al., 1989; Ben-Ari, 2002; Owens and Kriegstein, 2002). In contrast, in mature hippocampal neurons, the concentration of  $\text{Cl}^-$  is higher outside than inside the cells, and consequently  $\text{GABA}_A$ Rs mediate outward currents that hyperpolarize the cell membrane. It is currently thought that

the developmental shift of  $\text{Cl}^-$  gradient rely on the expression of different membrane transporters, which accumulate or extrude  $\text{Cl}^-$  in immature and mature neurons, respectively (Blaesse et al., 2009). The main transporters that regulate the levels of intracellular  $\text{Cl}^-$  have been identified; the NKCC1, which plays a key role in maintaining a high concentration of internal  $\text{Cl}^-$ , and the KCC2, which is the major extruder for  $\text{Cl}^-$  in neurons (Rivera et al., 1999; Blaesse et al., 2009). During development,  $\text{Cl}^-$  extrusion increases due to the downregulation of NKCC1 and upregulation of KCC2. Knowledge on the factors responsible for this transition is important, since  $\text{Cl}^-$  transporters ultimately determine the nature of GABAergic signalling. Surprisingly,  $\alpha 7$ -nAChRs were identified as a crucial key in defining the expression of  $\text{Cl}^-$  transporters (Liu et al., 2006). The mechanism used by  $\alpha 7$ -nAChRs is not totally clear, but is likely to involve a change in  $\text{Cl}^-$  transporter levels, making the equilibrium potential for  $\text{Cl}^-$  currents more negative. In other words,  $\alpha 7$ -nAChRs regulate the tempo for the conversion of GABAergic transmission from depolarizing to hyperpolarizing (Liu et al., 2006).

$\alpha 7$ -nAChR-mediated signaling also modulates the release of several transmitters in the developing hippocampus (Gray et al., 1996; Alkondon et al., 1997; Radcliff and Dani, 1998; Alkondon and Albuquerque, 2001). The facilitatory effect of  $\alpha 7$ -nAChRs on the release of glutamate and GABA can occur on multiple timescales, extending from seconds to days, and regulate important developmental phenomena that shape the neuronal network, like the spontaneous network events termed giant depolarizing potentials (GDPs) (Ben-Ari et al., 1989). The spiking of these neurons has been attributed to a synchronous, depolarizing GABAergic input from the interneuronal network acting in a synergistic manner with NMDA receptors. Activation of  $\alpha 7$ -nAChRs regulates the frequency of GDPs in the CA3 region of the hippocampus (Maggi et al., 2001, 2003; Le Magueresse et al., 2006) and the effect can be dramatic to the point that nicotine converts silent synapses to functional synapses. The mechanisms operated by  $\alpha 7$ -nAChRs in this process are still not known.

After the first two postnatal weeks, the occurrence of GDPs ceases. However,  $\alpha 7$ -nAChRs keep regulating the strength of synapses in young neurons by inducing the release of glutamate. nAChRs localized in mossy fibers boutons synchronize the release of glutamate in AP-independent form by increasing  $[\text{Ca}^{2+}]$  (Sharma and Vijayaraghavan,

2003). The increase in glutamate release is sufficient to drive the postsynaptic cell above the threshold for firing action potentials. This process of concerted release requires the downstream activation of presynaptic CaMKII demonstrating a novel, kinase-mediated, regulation of concerted release of multiple vesicles (Sharma et al., 2008). In addition,  $\alpha 7$ -nAChRs are expressed at the somatodendritic, preterminal and terminal levels of GABAergic neurons; they can depolarize the cell membrane (Alkondon et al., 2009) and eventually generate action potentials that lead to the release of GABA from presynaptic terminals (Alkondon et al., 1999).

Between postnatal day (P) 14 and P24, activation of  $\alpha 7$ -nAChRs can facilitate or inhibit to the induction of long-term plasticity, depending on the cell-type where they are expressed (Ji et al., 2001). Properly timed activation of  $\alpha 7$ -nAChRs at pyramidal neurons boosts the induction of long-term potentiation via presynaptic and postsynaptic pathways (Ji et al., 2001; Ge and Dani, 2005). On the other hand, nicotinic activity on interneurons inhibits nearby pyramidal neurons and thereby prevents or diminishes the induction of synaptic potentiation (Ji et al., 2001). The impact of  $\alpha 7$ -nAChRs in the induction of LTP of the intact brain is difficult to predict due to the diffuse cholinergic innervation and expression of different subtypes of nAChR in principal cells and interneurons. The expression of  $\alpha 7$ -nAChRs as well as their role in synaptic strengthening persist in the adult hippocampus, although it is not known yet if the same mechanisms are operated by  $\alpha 7$ -nAChRs in the developing and adult brain.

In the adult brain, nicotinic cholinergic input is also positioned well to influence adult neurogenesis. Early on, adult-born neurons receive cholinergic innervation and express two major types of ionotropic nAChRs: homopentameric  $\alpha 7$ -nAChRs and heteropentameric  $\beta 2$ -containing receptors ( $\beta 2^*$ -nAChRs) (Kaneko et al., 2006; Ide et al., 2008). Cholinergic forebrain lesion decreases adult-born neuron survival, and enhancing cholinergic activity increases survival (Cooper-Kuhn et al., 2004; Kaneko et al., 2006). Chronic nicotine exposure in vivo reduces adult-born neuron proliferation (Abrous et al., 2002; Shingo and Kito, 2005; Scerri et al., 2006), whereas  $\beta 2$ -nAChR KO mice show decreased proliferation, but normal survival, of hippocampal adult-born neurons (Harrist et al., 2004; Mechawar et al., 2004).

#### **1.4.6.2 Cholinergic signaling in pathology - the cholinergic hypothesis of Alzheimer's disease**

Although the role of nAChRs in the adult brain is not totally understood, they seem to be important for cognitive functions like learning and memory. Decline, disruption, or alterations of nicotinic cholinergic signaling are associated with several dysfunctions, including schizophrenia, epilepsy, autism, Alzheimer's disease, and addiction (Romanelli et al., 2007). One measure of normal age-related decline in the CNS is the diminishment and eventual dysfunction of the limbic cholinergic system that, in its most severe form, contributes to the neuropathologies of dementia, including Alzheimer's disease (Gouras et al., 2010). The histopathology of this disease is known for having at least four components: 1) loss of cholinergic neurotransmission, 2) deposition of amyloid beta ( $A\beta$ ) peptides into plaques, 3) hyperphosphorylation of the tau protein that leads to excessive formation of neurofibrillar tangles and 4) increased local inflammation (Gouras et al., 2010). Early deficits in Alzheimer's disease involve loss of cholinergic neurons and a diminution of cholinergic signaling (Whitehouse et al., 1982; Francis et al., 1999; Nordberg, 2001; Lyness et al., 2003). The  $A\beta$  accumulated during the disease (Selkoe, 1994) impairs choline uptake and acetylcholine release, further compromising cholinergic signaling (Auld et al., 1998; Kar and Quiron, 2004). Moreover,  $A\beta$  has been reported to inhibit  $\alpha 7$ -nAChR function either directly or indirectly (Wang et al., 2000a,b; Liu et al., 2001b; Pettit et al., 2001; Dougherty et al., 2003; Grassi et al., 2003; Lee and Wang, 2003; Pym et al., 2005), though it has also been reported to be an  $\alpha 7$ -nAChR agonist at low concentrations (Dineley et al., 2001, 2002; Dougherty et al., 2003; Grassi et al., 2003; Wang et al., 2003). Several studies have reported specific decrements in  $\alpha 7$ -nAChRs associated with Alzheimer's disease (Hellstrom-Lindahl et al., 1999; Guan et al., 2000; Lee et al., 2000; but see Reid et al., 2000). Currently, there is no cure for Alzheimer's disease. The medication usually prescribed for delaying the progression of Alzheimer's disease includes inhibitors of acetylcholine esterase, in order to prevent the cleavage of acetylcholine in the synaptic cleft and prolong its actions in the brain.



## **1.5 NEUROTROPHINS AS REGULATORS OF SYNAPTIC STRENGTH**

Neurotrophins constitute a class of molecules first described as important regulators of neurite outgrowth and sprouting, cell differentiation, migration and proliferation (Schinder and Poo, 2000; Vicario-Abejón et al., 2002; Nagappan and Lu, 2005). In addition to the classical effects, which are relatively slow, neurotrophins also elicit rapid signaling that modulates a variety of cellular functions such as membrane excitability, synaptic transmission, and activity-dependent synaptic plasticity in both peripheral and central neurons (Kang and Schuman, 1995; Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996; Lu et al., 2005). For the reasons stated above, neurotrophins have been considered regulatory molecules linking rapid changes at synapses with longer-lasting modifications of circuit activity.

The level of neurotrophins expression is high during development, but persists in many parts of the adult brain. Four mammalian neurotrophins have been characterized to date: nerve-growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). These factors are derived from a common ancestral gene and are largely similar in their structure and functions, hence the collective name neurotrophins (Lu et al., 2005).

Neurotrophins exert their cellular effects through the actions of two different receptors, the tropomyosin related-kinase (Trk) of tyrosine kinase receptor family and the pan-75 neurotrophin receptor (p75<sup>NTR</sup>), a member of the tumour necrosis factor receptor superfamily (Lu et al., 2005). Each neurotrophin has its specific Trk corresponding receptor: NGF binds TrkA, BDNF and NT4 bind TrkB, and NT3 interacts mainly with TrkC. All neurotrophins bind, however, the common p75<sup>NTR</sup> with a similar affinity. The role for these two separate receptor systems is currently understood as follows: the Trk receptors mediate survival signals emanating from the mature neurotrophins, whereas p75<sup>NTR</sup> mediates mainly apoptotic signals in response to pro-neurotrophins.

### **1.5.1 BRAIN DERIVED NEUROTROPHIC FACTOR**

Like all neurotrophins, BDNF is expressed in a region-specific manner in the nervous

system. Overall, the expression pattern of BDNF seems to be more widespread than that of the other neurotrophins in the adult CNS. The levels of BDNF mRNA rise dramatically during the first weeks after birth and BDNF remains abundantly expressed even in the adult brain, particularly in the cortex and hippocampus (Ernfors et al., 1990; Hofer et al., 1990; Maisonpierre et al., 1990).

One of the most intriguing characteristics of BDNF supporting its major role in the regulation of synaptic strength is that its gene translation and protein expression are highly controlled by neuronal activity (reviewed by Thoenen, 1995; Poo, 2001). Furthermore, the release of BDNF is significantly regulated by neuronal activity (Kuczewski et al., 2009) and curiously paradigms that elicit LTP are the most effective in inducing BDNF release (Hartmann et al., 2001; Aicardi et al., 2004). BDNF also increases its own release (Canossa et al., 1997), suggesting an important positive feed back loop in its signaling. Neuronal activity also elevates the responsiveness to BDNF by increasing available TrkB receptors in the cytoplasmic membrane via docking of TrkB-loaded intracellular vesicles (Castren et al., 1992; Meyer-Franke et al., 1998). Curiously, synaptic activity can inclusively induce TrkB activation without the presence of BDNF (Du et al., 2003). The findings described above might explain, at least in part, why BDNF signaling and function are restricted to active neurons/synapses.

#### **1.5.1.1 FAST ACTIONS OF BDNF IN THE HIPPOCAMPUS**

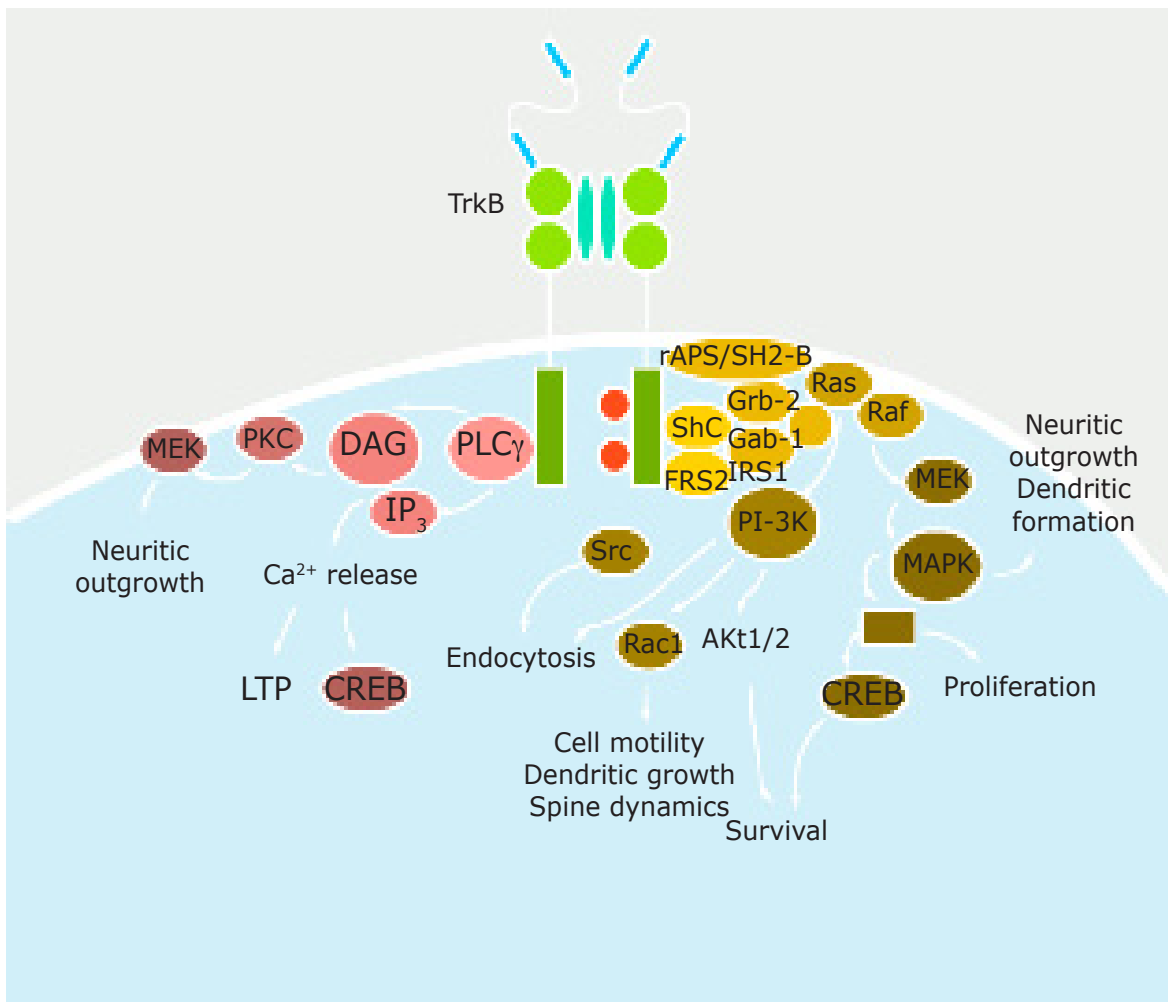
The fast actions of BDNF on synaptic function were first discovered at the NMJ in vitro. Application of BDNF to the neuromuscular synapses elicited a rapid enhancement of transmitter release (Boulanger and Poo, 1999a). At central synapses, BDNF has been reported to enhance excitatory synaptic transmission and suppress inhibitory transmission in both slice and dissociated cell cultures (Poo, 2001). In the hippocampus, TrkB receptors are expressed in both pyramidal neurons and GABAergic interneurons (Drake et al., 1999). In addition, TrkB labeling was also detected in some excitatory-type axon terminals resembling those known to arise from extrahippocampal afferents (Drake et al., 1999), which may contain GABA and/or neuromodulators. An interesting possibility is that these terminals themselves may be subject to modulation by TrkB

ligands (Abraham and Bear, 1996). Most synaptic effects of BDNF are accounted by presynaptic modification of transmitter secretion, probably due to BDNF-induced increase in cytosolic  $\text{Ca}^{2+}$  (Berninger et al., 1993; Stoop and Poo, 1996; Pozzo-Miller et al., 1999). In some instances, BDNF was found to modify the properties of postsynaptic transmitter channels, including AMPA, NMDA, and  $\text{GABA}_A$  receptors (Rose et al., 2004).

#### **1.5.1.2 Signaling mediated by TrkB Receptors**

TrkB receptors contain 10 evolutionarily conserved phosphorylation-regulated tyrosine residues in their intracellular domain (Huang and Reichardt, 2003). Upon ligand binding, TrkB receptors dimerize and become autophosphorylated in *trans* in their intracellular catalytic domain (Y701, Y705 and Y706) (Middlemas et al., 1994; Stephens et al., 1994; Segal et al., 1996). Autophosphorylation of the TrkB catalytic domain leads, in turn, to the phosphorylation of tyrosine residues in the juxtamembrane domain or the carboxyl terminus of the receptor, which act as docking sites for many of the intracellular enzymes and adaptors containing Src homology (SH2) or phosphotyrosine binding (PTB) domains (Segal et al., 1996; Huang and Reichardt, 2003).

Two of these tyrosines, Y515 and Y816, have been the major focus in elucidating Trk receptor signalling events. Phosphorylation of Y816 directly recruits and activates PLC1 through phosphorylation (Vetter et al., 1991; Middlemas et al., 1994). Activated PLC1 further hydrolyses phosphatidyl inositides to generate inositol 1,4,5 trisphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  increases  $[\text{Ca}^{2+}]_i$  by stimulating the release of  $\text{Ca}^{2+}$  from intracellular compartments and thus activates the signaling of a variety of  $\text{Ca}^{2+}$ -dependent intracellular molecules (e.g. CaM kinases). DAG activates DAG-dependent PKC isoforms, which can further regulate the extracellular signal-regulated kinase (ERK) signaling cascade. Phospho-Y515 serves as a docking site for Shc adaptor protein (Stephens et al., 1994), which through a series of intracellular events activates Ras-ERK and phosphatidylinositol-3-OH kinase (PI3K)-Akt/protein kinase B (PKB) cascades (Huang and Reichardt, 2003). Whereas the Ras-ERK pathway regulates neuronal differentiation, PI3k-Akt is important for cell survival (Huang and Reichardt, 2003). Another adaptor molecule, fibroblast growth factor receptor substrate 2 (FRS2), competes with Shc adaptor molecules for binding at this site. Additional adaptor proteins



**Figure 1.7 - Neurotrophin signaling pathways via TrkB receptor.** Abbreviations used: AKT, serine/threonine kinase; CREB, cAMP response element binding protein; DAG, diacylglycerol; FRS\_2, fibroblast receptor substrate-2; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; PI-3K, phosphatidylinositol-3-kinase; IP<sub>3</sub>, inositol-1,4,5-triphosphate; PLCγ, phospholipase Cγ.

containing pleckstrin homology (PH) and SH2 domains, such as SH2B and SH2B2, can associate with Trk receptors through direct binding to phosphotyrosine residues in the catalytic domain of the receptor. Activation of SH-2B further activates Grb2, which regulates the Ras-ERK and PI3K-Akt cascades (Qian et al., 1998).

Ras-ERK, Akt and PLC pathways can ultimately regulate the activation of CREB, an important transcription factor for many genes, whose products are implicated in neuronal plasticity, survival and neurotransmission (Mayr and Montminy, 2001). It is important to note that, in addition to Trk receptors, many intracellular signaling cascades (such as protein PKA or Ca<sup>2+</sup>-CaMK) can activate CREB. Some genes are only activated when the activation of these cascades simultaneously converge (Benito

and Barco, 2010), supporting the activity-dependent nature of CREB's action.

Besides the three major pathways described above and in Fig 1.7, neurotrophins also regulate the cytoskeleton in different cellular domains, e.g. the growth cones and the cell soma (Yuan et al., 2003). Rapid cytoskeletal dynamics induced by Trk activation are controlled by Rho, Rac and Cdc42 of the Rho family of small GTPases, leading to the assembly of contractile actin/myosin filaments, protrusive actin-rich lamellipodia, and protrusive actin-rich filopodia (Yuan et al., 2003).

#### **1.5.1.3 A ROLE FOR cAMP IN GATING TRKB-MEDIATED SIGNALING**

Substantial evidence indicates that 3'-5'-cyclic adenosine monophosphate (cAMP) is crucial for gating BDNF-mediated actions. Experiments using *Xenopus* nerve-muscle co-cultures provided some important new insights into the relationship between BDNF and cAMP (Boulanger and Poo, 1999b). Application of BDNF induced a rapid potentiation of transmission at neuromuscular synapses. Such potentiation exhibited the following features: (i) inhibitors of cAMP signaling blocked potentiation induced by high doses of BDNF; (ii) activators of cAMP signaling enhanced the potentiating effects of low-dose BDNF; and (iii) cAMP analogs alone did not mimic the BDNF effects. Thus, cAMP is not a downstream effector in the BDNF-mediated signaling cascade, but instead is permissive for the BDNF effect. Based on these experiments, cAMP was proposed to act as a 'gate' that enables BDNF to achieve its synaptic effects. Similar 'cAMP gating' features were described for other BDNF-mediated functions, including cell survival, growth cone turning, dendritic spine formation and hippocampal synaptic transmission (Meyer-Franke, 1995; Gaiddon et al., 1996; Song et al., 1997; Diógenes et al., 2004; Ji et al., 2005).

There are distinct mechanisms used by cAMP to modulate TrkB signaling in neurons. Early studies demonstrated that  $[cAMP]_i$  elevation enhanced the responsiveness of retinal ganglion cells to BDNF by increasing the surface expression of TrkB. Ji and colleagues demonstrated that cAMP modulates TrkB signaling in hippocampal neurons by: 1) regulating BDNF-induced TrkB tyrosine phosphorylation and 2) facilitating the movement of TrkB to PSDs of hippocampal neurons. More recently, it was described

that elevation of  $[cAMP]_i$  induces TrkB translocation to lipid rafts and potentiates TrkB receptor phosphorylation in these domains (Assaife-Lopes et al., 2010). Interestingly, this effect requires the presence of BDNF but it seems to involve different mechanisms from those used by BDNF to translocate TrkB receptors to lipid rafts (Suzuki et al., 2004; Pereira and Chao, 2006). The interaction between neurotrophins-dependent pathways and other coincident signals, including neuronal and synaptic activity, is also important for long-term trophic effects of gene activation (Benito and Barco, 2010).

The production of cAMP relies on the activation of adenylyl cyclase. One of the major contributors for activating adenylyl cyclase, and consequently, elevating  $[cAMP]_i$  levels in the neurons, is the adenosine  $A_{2A}$  receptor. Adenosine is a ubiquitous modulator of the nervous system acting via  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  G-protein-coupled receptors (GPCRs) (Fredholm et al., 2003; Sebastião and Ribeiro, 2009). Adenosine receptors are widely distributed in the nervous system; the high-affinity  $A_1$  and  $A_{2A}$  receptors are responsible for the fine-tuning the release of neurotransmitters and other signaling molecules (Sebastião and Ribeiro, 2009). The levels of extracellular adenosine at synapses are tightly regulated and fluctuate according to the rate of neuronal firing (Sebastião and Ribeiro, 2009). During high frequency neuronal firing, the release of the adenosine precursor ATP is increased (Wieraszko et al., 1989),  $A_{2A}$  receptor activation is favored (Correia-de-Sá et al., 1996), and adenosine inactivation systems are inhibited (Pinto-Duarte et al., 2005). In addition, ATP can inhibit ecto-5'-nucleotidase activity in the hippocampus, allowing the burst-like formation of adenosine (Cunha, 2001).

There are several evidences for a permissive role of adenosine  $A_{2A}$  receptors on the fast actions of BDNF upon synaptic transmission and LTP (Diógenes et al., 2004; Fontinha et al., 2009; Tebano et al., 2008; Assaife-Lopes et al., 2010). Furthermore,  $A_{2A}$  receptor activation not only triggers cAMP accumulation, but also activates PKC (Cunha and Ribeiro, 2000) and Src-family tyrosine kinases, suggesting the existence of parallel signaling systems that could gate BDNF actions (Rajagopal and Chao, 2006; Mojsilovic-Petrovic et al., 2006).

## 1.6 RATIONALE AND OBJECTIVES

As reviewed above, activation of nAChRs is able to produce diverse effects due to their location and downstream signaling pathways activated in cells. Specifically for  $\alpha 7$ -nAChRs, the  $\text{Ca}^{2+}$  influx can trigger different signaling cascades in neurons and some of them can inclusively regulate gene transcription.

It became also clear that there is limited knowledge on the intracellular pathways and extracellular signals that regulate the expression/function of neuronal nicotinic receptors on the cell membrane. Furthermore, the impact of nAChR-mediated signaling on the CNS is not totally clear yet. The aim of this dissertation is to investigate

**the mechanisms that regulate neuronal nAChRs on the cell surface and elucidate about the role played by these receptors in the central nervous system.**

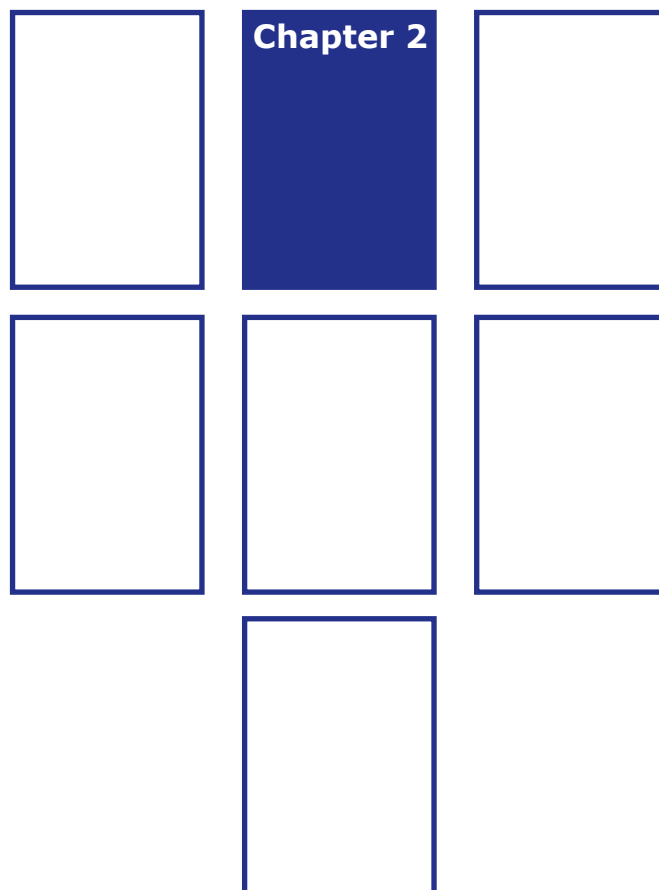
To accomplish these goals, I focused my work on the following objectives:

1. Characterize the lateral diffusion of neuronal nAChRs on the cell membrane and identify some cellular and molecular mechanisms that underlie the dynamic distribution of nAChRs on the cell surface.
2. Study the acute effect of the neurotrophin BDNF on the function of neuronal nAChRs.
3. Investigate the role of  $\alpha 7$ -nAChRs on the development of adult-born neurons in the dentate gyrus.

Fundamental information about the biology and cellular processes involved in cholinergic transmission will be gained. In addition, knowledge of these mechanisms may open new windows for understanding and developing new treatments for mental disorders, like Alzheimer's disease, schizophrenia and nicotine addiction.







## **MATERIALS AND METHODS**



## MATERIALS AND METHODS

### 2.1 ETHICS

All experiments were performed in accordance with European Community and National Institute of Health guidelines for animal care and use. Animals were maintained on a 12-hour light/dark cycle, and provided food and water *ad libidum*.

### 2.2 METHODS FOR CHAPTER III

#### 2.2.1 SINGLE PARTICLE TRACKING: IMAGING THE LATERAL DIFFUSION OF MEMBRANE MOLECULES WITH QUANTUM DOTS

To better understand the dynamic of receptors in the cell membrane, a variety of methods have been developed to optically track transmitter receptor movement, yielding important insight into the physical interactions and local environment of a receptor (Triller and Choquet, 2005; Groc et al., 2007). In the late 1980s, the emergence of SPT experiments allowed real-time monitoring of the movement of individual proteins or a small group of proteins on the plasma membrane (Geerts et al., 1987; Schnapp et al., 1988; Saxton and Jacobson, 1997). One of the main advantages of single-molecule imaging is that it avoids ensemble averaging of multiple molecules. As a result, it enables the observation of heterogeneous behaviors of a given molecule and of transient phenomena that take place.

In classical SPT experiments, the molecules of interest are specifically bound to high-affinity ligands, (such as antibodies, natural ligands, synthesized ligands or toxins), which in turn are attached to latex beads, colloidal gold nanoparticles, silica), that can be tracked by video microscopy with 10–100 Hz acquisition frequency and spatial resolution of 10–50 nm (Choquet and Triller, 2003). Several physical laws underpin the usefulness of SPT in reporting membrane protein diffusion. First, on this scale, mass is not a critical factor and movements are dominated by Brownian and viscous forces. Second, because the viscosity of membranes is 100- to 1000-fold greater than that of

extracellular fluids, it is the membrane-anchored receptors that slow down the particles and not the reverse. Third, the diffusion coefficient of an object in a membrane varies, in theory, as the logarithm of its surface area, because membranes are a two-dimensional diffusion space (Choquet and Triller, 2003).

Although SPT allows longer recording periods and better time resolution than other techniques, the size of the label can reduce or even prevent access to crowded areas and, for this reason, the use of SPT has been limited in neurons. For several years, the use of SPT was restricted to the study of extrasynaptic receptors, as the size of the particles prevented access to receptors to the synaptic cleft (Saxton and Jacobson, 1997). The recent development of fluorescent semiconductor QDs might partially overcome this difficulty and partially compensate the drawbacks of SPT. The total diameter of a QD is about 25–30 nm, making them suitable to track receptors inside the synaptic cleft (Dahan et al., 2003). Moreover, QDs offer unique photophysical properties that have provided new possibilities in biological imaging. Their bright fluorescence and resistance to photobleaching make it possible to achieve good signal-to-noise ratio, thereby increasing spatial resolution, and to track single particles for longer periods (the time resolution is mainly limited by the speed of the camera data transfer). Because they are defined point sources that can be imaged on large areas on charge-coupled devices, their localization can be determined with a relative resolution down to the nanometer scale. This provides an improved resolution compared to the usual resolution of a light-focusing microscope, which is limited to  $\lambda/2NA$  200–500 nm, where  $\lambda$  is the light wavelength and NA the numerical aperture of the lens. Such a molecular resolution has not been reached in approaches used to study membrane dynamics, such as fluorescence recovery after photobleaching (FRAP; Axelrod et al., 1976) or fluorescence correlation spectroscopy (Haustein and Schwille, 2004; Thoumine et al., 2008). This precision enables the determination of diffusion coefficients over several orders of magnitude. Finally, QDs have the particularity of alternating between 'on' and 'off' states, known as blinking (Michalet et al., 2005). This complicates the particle tracking but ensures the identification of single QDs because signals alternate between 0 and 1, and would be fractional in the case of multiple QDs. However, one cannot rule out the possibility that multiple receptors are bound to a single QD, and this point must

be considered carefully. It is usually impossible to precisely determine the number of receptors that are bound to an individual QD. Although theoretically this is not an issue, as diffusion of a small aggregate of receptors should be similar to that of an individual receptor, this only holds true if receptor aggregation facilitated by the particle does not trigger active mechanisms such as coupling to cytoskeleton elements. Furthermore, the complex microstructure of biological membranes probably impedes the diffusion of receptor aggregates more severely than diffusion of individual receptors.

Although luminescence properties of individual quantum dots should allow for their visualization in tissue, such as brain slices or in vivo, their size would likely limit their penetration, diffusion, or both. For this reason, the use of QDs for studying neurotransmitter lateral diffusion has been limited to cell cultures. Furthermore, attachment of these labels to native receptors usually relies on the availability of antibodies, which are rather bulky ligands, targeted to extracellular domains.

#### **2.2.1.1 PRIMARY NEURONAL CULTURE AND TRANSFECTION**

White leghorn chick embryos were obtained locally and maintained at 37°C in a humidified incubator. Dissociated E8 CG neurons were grown in culture for 6 days on glass bottom culture dishes coated with poly-D-lysine, fibronectin, and lysed fibroblasts at 2 ganglion equivalents per 16 mm coverslip (Nishi and Berg, 1981; Zhang et al., 1994). Under these conditions, the neurons form nicotinic cholinergic synapses on each other and express the same classes of receptors found in vivo (Chen et al., 2001; Conroy et al., 2003). Dissociated E13 chick sympathetic ganglion neurons from the lumbar region were grown in culture for 6 days on glass bottom culture dishes coated with poly-D-lysine at 1.5 ganglion equivalents per 16 mm coverslip. These growth conditions suppress the proliferation of nonneuronal cells resulting in cultures comprised of ~90% cells with neuronal morphology that are sensitive to applied acetylcholine (Downing and Role, 1987).

Neurons were transfected at the time of plating as described (Conroy et al., 2003) using the transfection reagent Effectene (0.25–0.5 µg DNA/well, 1:25 ratio of DNA/Effectene). The medium was replaced 24 hours after plating, and cultures were analyzed after 6 days. Typical transfection efficiencies were 1–2%.

### **2.2.1.2 PHARMACOLOGICAL MANIPULATIONS**

Actin filaments and microtubules were depolymerized by treating cultures for 1 hour at 37°C with latrunculin A (3  $\mu$ M) or cytochalasin D (2  $\mu$ M) and nocodazole (10  $\mu$ M), respectively, in 0.1% DMSO. For cholesterol depletion, cells were incubated for 2.5 hours with cholesterol oxidase (COase, 2 U/ml) or with methyl- $\beta$ -cyclodextrin (M $\beta$ CD, 3  $\mu$ M) for 15 min at 37°C.

### **2.2.1.3 SYNAPSE LABELING AND RECEPTORS TAGGING**

Functional synaptic specializations were labeled using either FM4-64 dye or MitoTracker Red 580 staining. FM4-64 is a red fluorescent amphiphilic styryl dye that embeds into the membranes of synaptic vesicles as endocytosis is stimulated; MitoTracker (rhodamine derivative) is a mitochondria marker that was shown to colocalize with the presynaptic synaptotagmin clusters (Tardin et al., 2003; Groc et al., 2004). FM4-64 (5  $\mu$ M) labeling was achieved by a 30 s incubation at RT with 40 mM KCl added to the culture medium to stimulate vesicle recycling. MitoTracker Red 580 (100 nM) staining was performed by adding the reagent to the culture medium for 2 minutes at 37°C, washing 2 times with culture media, and leaving the neurons an additional 30 minutes at 37°C.

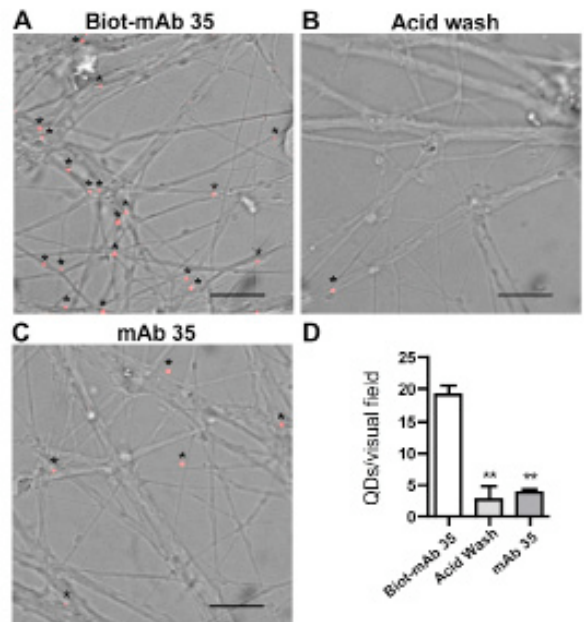
QDs tethered to individual  $\alpha$ 3\*- and  $\alpha$ 7-nAChRs via specific biotinylated ligands were used as fluorescent probes to follow the mobility of nAChRs. mAb 35 is a monoclonal antibody against one specific immunogenic region in  $\alpha$ 1,  $\alpha$ 3,  $\alpha$ 5 subunits of nicotinic acetylcholine receptors.  $\alpha$ -Bgt binds with high affinity to assembled  $\alpha$ 7-nAChRs receptors to a site that overlaps the agonist-binding site in a pseudo-irreversible fashion (because of the slow binding kinetics of  $\alpha$ -Bgt).

After rinsing three times with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, neurons were incubated with either biotinylated- $\alpha$ Bgt (Biot- $\alpha$ Bgt, 10 nM) or biotinylated mAb 35 (Biot-mAb 35, 0.1  $\mu$ g/ml) for 5 minutes on ice and washed three times. QDs 605 were tethered to individual  $\alpha$ 3\*- and  $\alpha$ 7-nAChRs by incubating neurons for 5 minutes with streptavidin-coated QDs (605 nm, 500 pM) on ice. Neurons

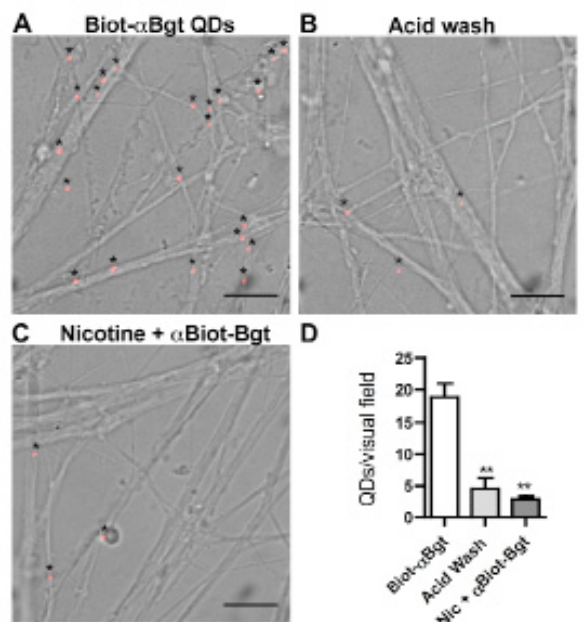
were washed three times with recording medium containing (in mM): 160 NaCl, 10 HEPES, 10 glucose, 4.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, pH 7.4. The labeling was specific because few QDs were observed for  $\alpha 3^*$ -nAChR labeling if the Biot-mAb 35 was replaced with unconjugated mAb 35 (Fig. 2.1C). Similarly, few QDs were observed for  $\alpha 7$ -nAChR labeling if the Biot- $\alpha$ Bgt was competed with 100  $\mu$ M nicotine (Fig. 2.2C). To minimize tracking internalized QD-nAChRs, all movies were confined to a 20-minute period immediately after the labeling (Charrier et al., 2006). Treating the cells with an acid wash [30 s with PBS pH 5.5 at room temperature (RT)] removed the vast majority of QDs, confirming that little, if any, internalization had occurred (Fig. 2.1D and 2.2D).

#### 2.2.1.4 ACQUISITION OF SPT MOVIES

Neurons were imaged with an inverted microscope (Zeiss Axiovert 200M) equipped with a 63x oil immersion objective (NA = 1.40). Samples were illuminated with a mercury lamp and imaged with appropriate excitation filters, dichroic mirrors, and emission filters. Settings were HQ545/30, Q565LP, and HQ610/75M, respectively for FM4-64 and



**Figure 2.1 - Images showing the number of QDs** (A) when using Biot-mAb 35 (0.1 mg/ml), (B) after acid-stripping 20 minutes following the staining shown in A, and (C) when using nonbiotinylated mAb 35. (D) Histogram represents the number of QDs in different conditions; \*\* $p < 0.01$ , one-way ANOVA. Scale bar: 10  $\mu$ m.



**Figure 2.2 - Images showing the number of QDs** (A) when using Biot- $\alpha$ Bgt (10 nM), (B) after acid-stripping 20 minutes following the staining shown in A, and (C) when using nonbiotinylated  $\alpha$ Bgt (10 nM). (D) Histogram represents the number of QDs in different conditions; \*\* $p < 0.01$ , one-way ANOVA. Scale bar: 10  $\mu$ m.

MitoTracker Red 580; D420/40X, 470DCXR, and D605/40, respectively, for QD 605; and HQ487/25, Q505LP, and HQ535/40M, respectively, for GFP. Fluorescence images were acquired with 95 ms exposure times at 10 Hz using a CCD camera (AxioCam MRm, Zeiss) and AxioVision 4.6 software (Zeiss).

#### **2.2.1.5 TRACKING PROCEDURE AND TRAJECTORY DECOMPOSITION**

QDs were only considered for analysis if they blinked during the recording period. Analysis was done as in Gómez-Varela et al., 2010. The trajectory (x,y,t) of each single particle was reconstructed. Image sequences were processed with the ImageJ 'SpotEnhancing Filter' plugin and tracking of single QD was performed with the ImageJ plugin (Sage et al., 2005). Periods where the fluorescence signal disappeared due to the blinking of single quantum dots were omitted from the analysis. In such cases, the trajectory was decomposed in two fragments ending and restarting immediately before and after the dark period of the blink respectively.

To assign synaptic localizations, trajectories were sorted into extrasynaptic and synaptic bins defined by the mitochondria marker MitoTracker Red 580 or the FM4-64 dye labeling. Synaptic spaces for MitoTracker and FM4-64-labeled boutons were defined as punctate regions 3 pixels in diameter (0.2  $\mu\text{m}$ /pixel; Aravanis et al., 2003). Synaptic and extrasynaptic trajectories were considered for 10 or more consecutive frames each, with the center of the QD inside the respective region.

#### **2.2.1.6 QUANTIFICATION OF DIFFUSION PROPERTIES**

Instantaneous diffusion coefficients ( $D_i$ ) were determined for each trajectory by fitting the first 5 points of the mean square displacement (MSD) curves versus the lag time ( $\tau_{\text{lag}}$ ; Dahan et al., 2003). MSD curves were constructed as described (Savin and Doyle, 2005), thereby rejecting the bias of the MSD resulting from diffusive motion during the exposure time of a single image:

$$\text{MSD}(\tau_{\text{lag}}, \tau_{\text{exp}}) = 4 D_i (\tau_{\text{lag}} - \tau_{\text{exp}} / 3) + d_{\text{loc}}$$



where  $\tau_{\text{exp}}$  represents the sum of all single exposure times applied for acquisition from  $t = 0$  to  $t = \tau_{\text{lag}}$  and corrects for the underestimation of  $D_i$  that becomes relevant if the exposure time  $\tau_{\text{exp}}$  is comparable to  $\tau_{\text{lag}}$ . The global localization accuracy ( $d_{\text{loc}}$ ) was determined by testing the vibrational stability of the setup, calculating MSD for immobile spots. Immobile QDs were defined as streptavidin-QDs stationary on the surface of glass bottom dishes in the absence of neurons. The localization accuracy was 50 nm, and the resolution limit in terms of diffusion coefficients was  $0.008 \mu\text{m}^2/\text{s}$ . Synaptic dwell time was calculated as the total time spent by a QD-nAChR in synaptic space divided by the number of exits by the QD from the space. Dwell time is related to the stability of the molecular interactions and/or to confinement in a given compartment. The frequency of transitions represents the sum of entries and exits from synaptic space divided by the duration of the recording period. Transition frequencies are related to the statistics of the discrete molecular events involved and whether or not they are stationary.

### **2.2.2 IMMUNOSTAINING OF FIXED CELLS**

To label surface  $\alpha 3^*$ -nAChRs, CG neurons in culture were lightly fixed with 0.15% paraformaldehyde (PFA) for 20 minutes at RT, washed in PBS, and incubated with mAb 35 (1:200) for 1 hour at RT (Conroy and Berg, 1998). A 45-minute incubation with rhodamine- $\alpha$ Bgt (100 nM) was used to label  $\alpha 7$ -nAChRs prior to fixation as previously described (Conroy et al., 2003). After washing in PBS, cells were then fixed with 2% PFA in PBS for 20 minutes at RT. To label synaptic boutons, cells were incubated with the anti-synaptotagmin mAb 48 (1:20) and anti-synaptic vesicle protein 2 (SV2) antibody (1:1000) overnight at 4°C in PBS containing 5% normal donkey serum and 0.1% Triton X-100. After washing in PBS, cells were incubated with appropriate donkey Cy3- or FITC-conjugated secondary antibody 1 hour at RT (1:250), rinsed, and mounted on slides for imaging.

### **2.2.2.1 FLUORESCENCE IMAGE ACQUISITION AND QUANTITATIVE ANALYSIS**

Confocal images were acquired in sequential mode using a Leica SP5 confocal microscope with settings that did not saturate the fluorescence signals and that fulfilled Nyquist sampling criteria. ImageJ software was used for quantifying the labeling intensity and the alignment of receptor clusters with SV2/synaptotagmin puncta. For this analysis, regions of interest (ROIs) of 20  $\mu\text{m}$  were selected on the neurites. ROIs were binarized automatically using the mean of the background value inside the neurite plus two times the standard deviation as an intensity threshold value for defining a cluster or puncta in each image. Clusters/puncta within the ROIs were registered if they had at least 3x3 pixels above threshold (pixel diameter: 80 nm). Receptor clusters were considered synaptic if they were  $\leq 2$  pixels away from SV2/synaptotagmin puncta. Data are expressed as the mean  $\pm$  SEM per neurite length of 10  $\mu\text{m}$ .

### **2.2.3 STATISTICAL ANALYSES**

Analyses were conducted with the Prism version 4.00 (GraphPad Software). For SPT experiments, statistical analysis was assessed with Mann–Whitney U test (MW test) for comparisons of diffusion coefficients if single pairs were involved; Kruskal-Wallis test (KW test) was used for  $\geq 3$  values. Student's t test (t test) was used for comparisons of dwell-times and numbers of transitions if single pairs were involved; one-way ANOVA was used for  $\geq 3$  values. The n values report the number of QDs or trajectories examined. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

For the immunostaining of fixed cells, Student's t test (t test) was used for comparisons of the number, size of clusters, pixel intensity and colocalization of receptor clusters with presynaptic markers; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## **2.3 METHODS FOR CHAPTERS IV AND V**

### **2.3.1 PATCH CLAMP TECHNIQUE IN TISSUE SLICES**

The patch clamp technique allows the electrophysiological study of single or multiple

ion channels and transmitter receptors in cells. This technique can be applied to a wide variety of cells, but is especially useful when studying excitable cells such as neurons (Sakmann and Neher, 1994). In the present work, we used 1) whole cell patch clamping, which was employed to measure the average current across the entire surface area of one cell, and 2) perforated patch clamping, which was used to minimize the dialysis of intracellular components.

In the patch clamp technique, a glass micropipette with an open tip (diameter around 1  $\mu\text{m}$ ) contacts the membrane surface; gentle suction leads a membrane patch to get enclosed into the pipette and a gigaOhm seal is formed (Hamill et al., 1981). The high resistance of the seal permits to electronically isolate the currents measured across the membrane patch with little competing noise, as well as providing some mechanical stability to the recording. The whole-cell configuration can be achieved by adding additional suction; the patch is ruptured, creating electrical continuity between the pipette and cell interior.

In whole-cell recordings, the interior of the pipette is filled with an internal solution that eventually replaces the intracellular fluid. Although this mechanism can be used advantageously in many experiments, there are conditions where such a dialysis interferes with response to be tested, since the “washout” of intracellular biochemical machinery may affect electrophysiological properties. For example, the response can disappear sometimes within minutes (Rose et al., 2003), an event that is usually called “rundown”. To overcome these problems, several strategies have been developed in the last decades; one of them is the perforated patch clamp technique, in which pore-forming antibiotics such as nystatin, amphotericin B or gramicidin are added to the internal solution (Zhao et al., 2009). As the antibiotic molecules diffuse into the membrane patch, they form small pores in the membrane, providing electrical access to the cell interior. Although the pores are permeable to small monovalent ions, they cannot be trespassed by larger ions and molecules, including soluble second messengers. This has the advantage of reducing the dialysis of the cell that occurs in whole-cell recordings and prevents the “rundown” of some electrophysiological responses. Gramicidin, in particular, is extremely useful for measuring the natural reversal potential for  $\text{Cl}^-$  in neurons. The pores formed by gramicidin are impermeable to negatively charged ions,

leaving the natural  $\text{Cl}^-$  cytoplasmic content undisturbed during the recording (Ebihara et al., 1995; Kyrozis and Reichling, 1995).

Perforated patch clamp technique also has some disadvantages. First, the access resistance is higher, relative to whole-cell, due to the partial membrane occupying the tip of the electrode (access resistance being the sum of the electrode resistance and the resistance at the electrode-cell junction). This will decrease electrical access and thus decrease current resolution, increase recording noise, and magnify any series resistance error. Second, it can take a significant amount of time for the antibiotic to perforate the membrane (10–30 minutes, though this can be reduced with properly shaped electrodes). Third, the membrane under the electrode tip is weakened by the perforations formed by the antibiotic and can rupture. If the patch ruptures, the recording is then in whole-cell mode, with antibiotic contaminating the inside of the cell.

Independently on the patch clamp configuration, there are two different recording modes – the current clamp and the voltage clamp (Sakmann and Neher, 1994). In a **current-clamp** (CC) experiment, a known constant or time-varying current is applied to the cell and the resulting change in membrane potential is measured. This type of experiment mimics the current produced by a synaptic input. In **voltage-clamp** (VC) experiments, the membrane voltage is kept constant during the recordings and the transmembrane current is measured. Despite the fact that voltage clamp does not mimic a process found in nature, there are three reasons to do such an experiment: 1) clamping the voltage eliminates the capacitive current, except for a brief time following a step to a new voltage, the currents that flow are proportional only to the membrane conductance, i.e., to the number of open channels (except for the brief charging time), 3) if channel gating is determined by the transmembrane voltage alone, voltage clamp offers control over the key variable that determines the opening and closing of ion channels.

### **2.3.1.1 HIPPOCAMPAL SLICES PREPARATION**

Cells in tissue slices are likely to be much closer to their original state. No disruption

of the normal cellular environment need take place until the preparation of slices and disruption is limited to the surface of the slice.

For the experiments described in **Chapter IV**, rats were anesthetized with halothane before decapitation, the brains were removed, hemisected and trimmed to contain a block of tissue surrounding the hippocampus. Transverse hippocampal slices (300  $\mu\text{m}$  thick) from 3-4 weeks old male Wistar rats (Harlan) were cut on a vibratome in an ice-cold solution containing (in mM) sucrose 110, KCl 2.5,  $\text{CaCl}_2$  0.5,  $\text{MgCl}_2$  7,  $\text{NaHCO}_3$  25,  $\text{NaH}_2\text{PO}_4$  1.25, glucose 10, bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ , pH 7.4. Slices were then incubated in artificial cerebrospinal fluid containing (aCSF, in mM) NaCl 124, KCl 3;  $\text{NaH}_2\text{PO}_4$  1.25,  $\text{NaHCO}_3$  26,  $\text{MgSO}_4$  1,  $\text{CaCl}_2$  2, glucose 10, pH 7.4, and equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  at 35° C for 30 min and afterwards maintained at room temperature (22-24° C) for at least 1h before use.

For experiments described in **Chapter V**, mice had C57Bl/6 background and used at 1 month of age. The  $\alpha 7\text{KO}$  mice were purchased from Jackson Laboratories, bred as heterozygotes, and genotyped by PCR. Animals were decapitated after being anesthetized by intraperitoneal injection of 10 mg/ml ketamine and 1 mg/ml xylazine in sterile 0.9% NaCl at a volume of 0.01 ml/g body weight. The brain was quickly removed from the skull and placed in an ice-cold solution containing (in mM): sucrose 75, NaCl 87, KCl 2.5,  $\text{CaCl}_2$  0.5,  $\text{MgCl}_2$  7,  $\text{NaHCO}_3$  25,  $\text{NaH}_2\text{PO}_4$  1.25, glucose 20, bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ , pH 7.4. Transverse hippocampal slices (250  $\mu\text{m}$  thick) were cut using a vibratome (series 1000 Plus) and stored at 30° C for 30 min in artificial cerebrospinal fluid containing (aCSF, in mM): NaCl 119, KCl 2.5;  $\text{NaH}_2\text{PO}_4$  1,  $\text{NaHCO}_3$  26,  $\text{MgCl}_2$  1.3,  $\text{CaCl}_2$  2.5, glucose 10, pH 7.4, and equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . After a recovery period of at least 1 h at room temperature (22-24° C), an individual slice was transferred to the recording chamber, in which it was continuously superfused with oxygenated aCSF at a rate of 2–3 ml/min.

### **2.3.1.2 PATCH CLAMP RECORDINGS**

Individual slices were fixed on a grid in a recording chamber for submerged slices and

continuously superfused at 3 ml.min<sup>-1</sup> with aCSF solution at room temperature.

In **Chapter IV**, whole-cell recordings were obtained from interneurons located at the CA1 *stratum radiatum* and at the border of the *strata radiatum* and *lacunosum moleculare*. Cells were visualized with an upright microscope (Zeiss Axioskop 2FS) equipped with infrared video microscopy and differential interference contrast optics. Cells were held at a membrane potential of -70 mV and recordings were performed at room temperature (22–24° C). Responses were recorded using a List EPC7 amplifier, filtered at 10 and 3 kHz through a three-pole Bessel filter and digitized at 5 kHz with WinLTP software (Anderson and Collingridge, 2007). Pipettes resistances were 5–7 MΩ. Series resistance was measured by the instantaneous current response to a -1 mV step with the pipette capacitance cancelled. Offset potentials were nulled directly before formation of a seal. Small voltage steps (1 mV, 50 ms) were evoked to monitor membrane and series resistances; if one of both or holding current changed significantly, the experiment was rejected. Junction potentials and voltage errors due to series resistance were not corrected. The internal solution consisted of (in mM): potassium gluconate 125, KCl 11, CaCl<sub>2</sub> 0.1, MgCl<sub>2</sub> 2, EGTA 1, HEPES 10, NaATP 2, NaGTP 0.3 and TRIS phosphocreatine 10, pH 7.3 adjusted with KOH, 280–290 mOsm. For perforated-patch recordings, gramicidin was diluted in the filling solution to a final concentration of 100 µg.ml<sup>-1</sup>. The tip of the electrode was filled with gramicidin-free pipette solution. In some experiments (where indicated) K252a (200nM), H-89 (0.1–1 µM), U73122 (5 µM), PP2 (100 nM), GF 109203X (2 µM) or Cytochalasin D (5 µM), dissolved in dimethyl sulfoxide (DMSO; 0.1%), were included in the internal solution; matching controls were performed with an equal percentage of DMSO in the internal solution.

α7-nAChR-mediated currents were evoked using a pressure ejection system (PicoPump PV820). A patch pipette containing acetylcholine (ACh) (1 mM) or choline (Ch) (10 mM) was positioned near the cell bodies and pulses of pressure were applied (5–10 psi, 30 ms, each 3 min). Stable baselines of 30 min, at least, were obtained before starting each trial. All recordings were performed in the presence of TTX (1 µM), CNQX (25 µM), APV (10 µM) and bicuculline (20 µM). Experiments were rejected if the superfusion of selective α7-nAChR antagonists, MLA (10 nM) or BGTx (100 nM), failed to completely

block  $\alpha 7$ -nAChR-mediated currents at the end of the trials.

In **Chapter V**, granule cells were visualized with an upright microscope (Zeiss Axioskop) equipped with differential interference contrast optics and fluorescence microscopy. GFP<sup>+</sup> granule cells (see under) were identified in the granule cell layer by their green fluorescence and their neuronal morphology.

The whole-cell patch-clamp configuration was employed in voltage-clamp or current-clamp modes. Microelectrodes (5–8 M $\Omega$ ) were pulled from borosilicate glass capillaries (Sutter Instruments) with a P-97 pipette puller (Sutter Instruments).

To record spontaneous synaptic currents (SSCs), the electrodes were filled with an internal solution containing (in mM): CsCl 135, MgCl<sub>2</sub> 4, EGTA 0.1, HEPES 10, NaATP 2, NaGTP 0.3 and Na<sub>2</sub>phosphocreatine 10, pH 7.4 adjusted with CsOH, 280–290 mOsm. GFP<sup>+</sup> granule cells were held at a membrane potential of -80 mV and five minutes of continuous sweeps were recorded. The resting membrane potential was determined in current clamp mode immediately after establishing the whole-cell configuration. The internal solution consisted of (in mM): K-gluconate 125, KCl 15, NaCl 8, EGTA 2, HEPES 10, MgATP 4, NaGTP 0.3 and Na<sub>2</sub>phosphocreatine 10, pH 7.3 adjusted with KOH, 280–290 mOsm. The criteria to include cells in the analysis was absolute leak current <100 pA at V<sub>Hold</sub>.

Perforated patch recordings with gramicidin were used for measuring the polarity of GABAergic transmission. The gramicidin stock (5 mg/ml in DMSO) was diluted in the pipette solution (in mM: 135 CsCl, 4 MgCl<sub>2</sub>, 0.1 EGTA, 10 HEPES, pH 7.4 adjusted with CsOH, 300 mOsm) to a final concentration of 50  $\mu$ g/ml just before the experiments. The tip of the electrode was filled with gramicidin-free pipette solution. Pipettes resistances were 5–8 M $\Omega$ . Small voltage steps (-10 mV, 50 ms) were evoked to monitor membrane and access resistances; if one of both or holding current changed significantly, the experiment was rejected. Since the collapse of the chloride gradient by strong extracellular stimuli or GABA applications could account for previously reported conflicting results regarding the polarity of GABAergic transmission, we electrically stimulated the release of endogenous GABA and recorded the response in adult-born neurons identified by MMLV labeling (see under). Extracellular stimulation (80–240  $\mu$ A and 0.2 ms duration, 0.1 Hz) was done using a concentric bipolar electrode (125  $\mu$ m diameter), with a pulse

generator coupled through a stimulus isolator. The stimulation electrode was placed on the granule cell layer, around 300  $\mu\text{m}$  away from the recorded cell. Evoked GABA-mediated postsynaptic currents (GPSCs) were pharmacologically isolated by adding glutamatergic blockers, CNQX (20  $\mu\text{M}$ ) and AP-5 (20  $\mu\text{M}$ ), to the bathing solution. GPSCs were recorded under voltage-clamp at different holding potentials. The peak amplitude and holding potential were plotted and the reversal potential ( $E_{\text{GABA}}$ ) was calculated from the linear fit of the I-V curve for each cell. The reversal potential of a current mediated by an ion channel corresponds to the electrochemical equilibrium for that current (no net movement of ion takes place, despite the channels are opened for electrodiffusion) and is given by the Nernst equation:

$$E = (RT/zF) \ln(\text{conc}_{\text{out}}/\text{conc}_{\text{in}}),$$

where R is the gas constant (8.314 J/mol K); T is the temperature (K); z is the valence for the ion; F is Faraday's constant (96500 C/mol); E is in volts.

As a control for perforated-patch integrity, at the end of some experiments, the  $E_{\text{GABA}}$  was calculated in the whole-cell configuration. The estimated  $E_{\text{GABA}}$  for the internal solution used was 2.7 mV. Current kinetic measurements were made from >5 averaged traces acquired at -80 mV holding potential. Current decay was determined between 10% and 90% of peak amplitude. Since the best fit for decay was either one or two exponentials, weighted decay was calculated using the equation  $A_1\tau_1 + A_2\tau_2$  where A is the relative amplitude and  $\tau$  is the decay constant for each component.

Data was collected using a MultiClamp 700A amplifier (Axon), filtered at 2 kHz and digitized at 5 kHz with pCLAMP 9 software (Molecular devices). Analysis was performed with the Clampfit 9.2 software (Molecular devices).

### **2.3.1.3 STATISTICAL ANALYSIS**

Analyses were conducted with the Prism version 4.00 (GraphPad Software). Results are expressed as the mean  $\pm$  SEM of n experiments. Statistical significance was assessed by Student's t test if single pairs were involved. One-way ANOVA followed by the Tukey-



Kramer post hoc test was used for comparing multiple groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

### **2.3.2 IDENTIFICATION OF ADULT-BORN NEURONS IN THE GRANULE CELL LAYER**

The granule cell layer is constituted by a heterogeneous population of cells with different degrees of cellular maturity due to the daily generation of new neurons from stem cells in the hippocampus.

To identify and birthdate adult-born neurons in the DG, retroviral vectors that express green fluorescent protein (GFP) have been stereotactically injected *in vivo* in the adult hippocampus. Retrovirus can only infect dividing cells; once incorporated into progenitors during mitosis, subsequent newborn cells express GFP and can be visualized in living preparations. Labeling is largely confined to neurons born within a 3-day window following virus injection (Zhao et al., 2006). This method has been applied for examining electrophysiological and morphological details in newborn neurons throughout their lifetime (van Praag et al, 2002).

The bromodeoxyuridine (BrdU) is a thymidine analogue that is incorporated into the DNA of dividing cells and can be detected immunohistochemically in their progeny (Nowakowski et al., 1989). Since BrdU is incorporated by dividing cells, including by the neuronal precursors in the granule cell layer (Kempermann et al., 2007a,b) its labeling can be used to assess proliferation of a population of cells or to assess the survival of labeled cells.

#### **2.3.2.1 STEREOTAXIC VIRAL INJECTION**

A Moloney's Murine Leukemia Virus construct expressing GFP (MMLV-GFP) was provided by Fred Gage (Salk Institute). Viruses were generated by transfecting the constructs into HEK293T cells. Harvest and concentration by ultra-centrifugation generated viral titers of 10<sup>8</sup> pfu/ml in sterile PBS. The viral suspensions were stereotactically delivered as described (Van Praag et al., 2002) at the following positions from Bregma: anteroposterior, -2mm; lateral, 1.7mm; ventral, -2mm; anteroposterior, -2.5mm; lateral, 2mm; ventral -2.2mm. Animal body temperature was maintained throughout

the surgery until anesthesia wore off. After surgery, animals were housed singly and monitored to ensure no signs of infection, pain, or distress.

#### **2.3.2.2 BrdU labelling**

5-bromo-2-deoxyuridine (BrdU) was injected intraperitoneally at 10 mg/ml in sterile 0.9% NaCl to yield a single dose of 50 µg/g body weight on each of 4 consecutive days. Mice were singly housed for 2 or 4 weeks after the initial injection until tissue preparation. After perfusion, all steps were performed blind to genotype. After cryostat sectioning, slices were dipped in 2N HCl for 30 min at 37°C, followed by 0.1 M borate buffer for 10 min at RT. After immunostaining, counts were made of BrdU-positive cells in the first third of the granule cell layer in every fourth section through the entire hippocampus. Adult-born neurons are mostly confined to the first third of the granule cell layer (Zhao et al., 2006).

#### **2.3.2.3 Imaging and quantification**

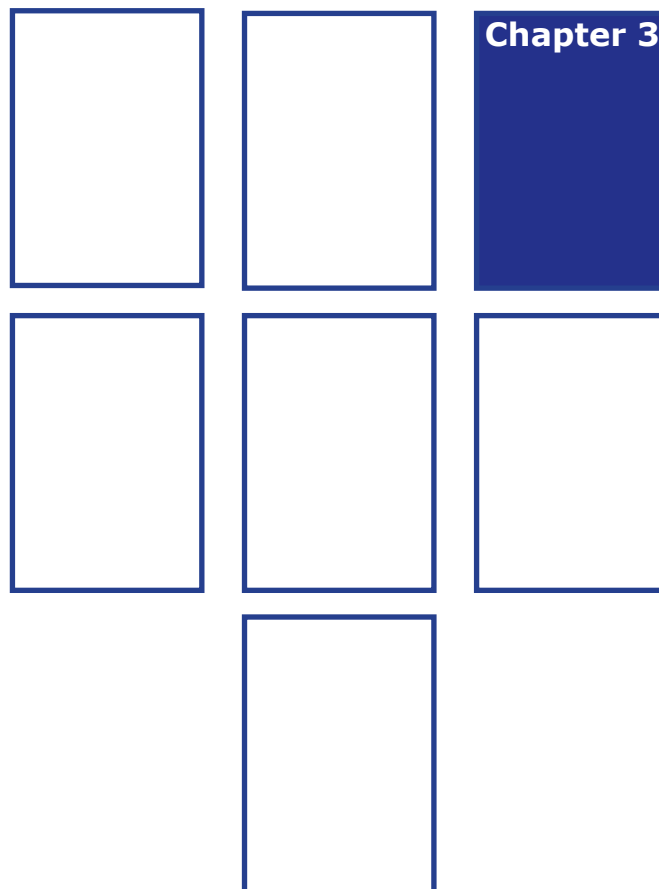
Imaging was performed within 48 h of immunostaining using a Zeiss Axiovert microscope with 3I deconvolution software for image analysis. For morphological measurements neurons were imaged at 63x magnification, and a z-stack was compiled by acquiring images every 0.5 µm through the section. Dendritic measurements were made in ImageJ using the NeuronJ tracing application. Spine counts were taken over a 20 µm segment of dendrite located within 100 µm of the granule cell layer boundary. Cell selection and quantification were performed blind to genotype.

### **2.4 CHEMICALS**

Streptavidin Quantum dot 605 conjugated (QDs), MitoTracker Red 580, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64), Biotinylated  $\alpha$ -Bungarotoxin (Biot-Bgt), rhodamine- $\alpha$ -Bungarotoxin and 5-bromo-2-deoxyuridine (BrdU) staining kit were from Invitrogen. mab48 and anti-SV2 were from Developmental Studies Hybridoma Bank. Cy3- or FITC-conjugated

secondary antibodies were from Jackson ImmunoResearch Laboratories. Effectene was from Qiagen. 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX), 2-amino-5-phosphonovalerate (APV), bicuculline methochloride, tetrodotoxin citrate (TTX), gabazine, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261), U 73122, PP2 and GF 109203X were from Tocris Bioscience, UK. Latrunculin A (LAT), cytochalasin D (Cyt D), nocodazole (NZ), cholesterol oxidase (COase), methyl- $\beta$ -cyclodextrin (M $\beta$ CD), acetylcholine chloride (ACh), choline chloride (Ch), methyllycaconitine citrate (MLA), dihydro- $\beta$ -erythroidine hydrobromide (dh $\beta$ e), phorbol-12,13-didecanoate (PDD), N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89) and gramicidin were from Sigma-Aldrich.  $\alpha$ -Bungarotoxin ( $\alpha$ -BT) and K-252a were obtained from Calbiochem-Merck Biosciences Ltd., UK. Cholesterol oxidase (COase) was provided by Sigma in 10 mM Tris-HCl (pH $\approx$ 8). Adenosine deaminase (ADA) was provided by Roche in a 200 U.ml<sup>-1</sup> stock solution in 50% glycerol (v/v) and 10 mM potassium phosphate (pH 6). Brain-derived neurotrophic factor (BDNF) was kindly provided by Regeneron Pharmaceuticals in a 1.0 mg.ml<sup>-1</sup> stock solution in 150 mM NaCl, 10mM sodium phosphate buffer and 0.004% Tween-20. Inactivated BDNF (HI-BDNF) was prepared by heating aliquots to 100°C for 30 min. ACh (0.5 M), Ch (0.5 M), TTX (1 mM), APV (25 mM), MLA (10 mM),  $\alpha$ -BT (20 nM) and dh $\beta$ e (10 mM) were prepared as stock solution in water. LAT (3 mM), Cyt D (2 or 5 mM), NZ (10 mM), CNQX (100 mM), bicuculline (100 mM), CGS 21680 (5 mM), SCH 58261 (5mM), H-89 (5mM), K252a (1 mM), PP2 (20 mM), U 73122 (5 mM), GF 109203X (1 mM), PDD (1 mM) and gramicidin (5 or 100 mg.ml<sup>-1</sup>) were prepared as a stock solution in DMSO. The percentage of vehicle (DMSO) in each experiment did not exceed 0.1%. Stock solutions were aliquoted and stored at -20°C, except for BDNF that was stored at -80° C, and aqueous dilutions of these stock solutions were made freshly before the experiment.





**LATERAL MOBILITY OF NICOTINIC RECEPTORS ON PERIPHERAL NEURONS IS  
DETERMINED BY RECEPTOR COMPOSITION, LOCAL DOMAIN AND CELL TYPE**



## **LATERAL MOBILITY OF NICOTINIC RECEPTORS ON PERIPHERAL NEURONS IS DETERMINED BY RECEPTOR COMPOSITION, LOCAL DOMAIN AND CELL TYPE**

### **3.1 INTRODUCTION**

The high concentration of neurotransmitter receptors within the postsynaptic specialization led to the predominant view that synaptic receptors are tightly fixed within the synapse. Over the last 40 years, however, increasing evidences have shown that synaptic complexes, including neurotransmitter receptors, are highly dynamic and can rapidly exchange overtime (Newpher and Ehlers, 2008).

The first evidences showing that nAChRs are mobile were found in the NMJ. Imaging of nAChRs with fluorescently labeled  $\alpha$ -Bgt showed that receptors “spontaneously” integrated into newly assembled NMJs during development, providing clear evidence for the aggregation of extrajunctional receptors in the nascent NMJs (Anderson and Cohen, 1977). Furthermore, the population of muscle nAChRs was not homogeneous, since it was possible to discriminate two different populations of nAChRs in the surface; one that could exist freely moving diffusely and other that was relatively immobile contained within concentrated patches (Axelrod et al., 1978). Importantly, the relatively immobile nAChRs contained in patches could be dispersed by electrical stimulation (Axelrod et al., 1978), showing that receptor aggregation is directly affected by synaptic activity. Besides having a fundamental role in receptor aggregation, lateral diffusion of functional nAChRs was found to have a central role in replacing inactivated receptors, promoting the recovery of nAChRs-mediated responses at sites of local inactivation (Young and Poo, 1983). Taken together, these studies showed that muscle nAChRs exchange between distinct membrane microdomains through a process involving lateral diffusion in the plasma membrane.

In neurons, transmitter receptors show lateral diffusion in the cell membrane as well, allowing them to exchange between extrasynaptic and synaptic sites in a short

time scale. A variety of methods have been developed to optically track glutamate receptors movement, yielding important insight into the physical interactions and local environment of a receptor (Groc et al., 2007; Triller and Choquet, 2008).

Rapid trafficking of neuronal nAChRs in the surface membrane is only beginning to be examined. A recent study has shown that neuronal nAChRs on autonomic neurons are capable of rapid lateral diffusion into and out of synapses in vivo (McCann et al., 2008). How this trafficking is regulated and what role it might play in nicotinic signaling is still a matter to be studied.

In the results section of this chapter, single-particle-tracking techniques with QDs (Heine et al., 2008b; Triller and Choquet, 2008) were used to follow the diffusion of nAChRs within the cell membrane of ciliary ganglion neurons in culture. The neurons form nicotinic cholinergic synapses onto each other under these conditions and express the same two classes of receptors found in vivo: homopentameric  $\alpha 7$ -nAChRs and heteropentameric  $\alpha 3^*$ -nAChRs (Margiotta and Berg, 1982; Vernallis et al., 1993; Chen et al., 2001). Both  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs are found at synaptic contacts in CG cultures, but  $\alpha 3^*$ -nAChRs generate most of the synaptic response (Chen et al., 2001; Conroy et al., 2003; Neff et al., 2009b). There is little cellular diversity within this culture system, which was one of the attractive reasons for using this preparation in our study.

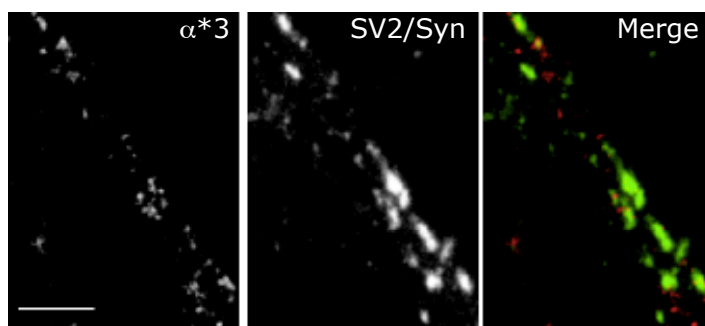
**In this chapter, the lateral diffusion of endogenous  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs was characterized; how the cytoskeleton, lipid rafts and PDZ-containing scaffolds regulate the lateral diffusion of these receptors was then investigated.**

## **3.2 RESULTS**

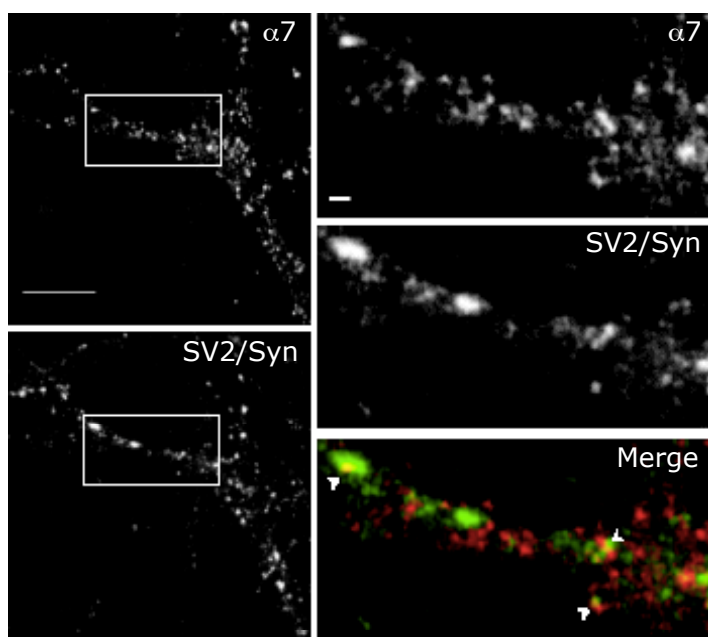
### **3.2.1 EXPRESSION OF nAChRs ON NEURITES OF CG NEURONS IN CULTURE**

To study the subcellular distribution of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs on the neuritis of CG neurons in culture, cells were fixed and immunostained for nAChRs and for the presynaptic markers SV2 and synaptophysin.  $\alpha 3^*$ - were visualized in both synaptic and extrasynaptic regions (Fig 3.1). Though  $\alpha 7$ -nAChRs are thought to be excluded from synaptic regions on adult CG neurons in vivo (Williams et al., 1998; Shoop et al., 1999), in cell cultures.  $\alpha 7$ -nAChRs were also localized on neurites at synaptic sites





**Figure 3.1 – Expression of  $\alpha 3^*$ -nAChRs in ciliary ganglion neurons.** Images of  $\alpha 3^*$ -nAChRs clusters ( $\alpha 3$ ; red in merge) on neurites in juxtaposition to presynaptic structures revealed by the SV2/synaptophysin immunostaining (SV2/Syn; green in merge). Arrows indicate examples of receptor-presynaptic alignment. Scale bar: 5  $\mu$ m (left).



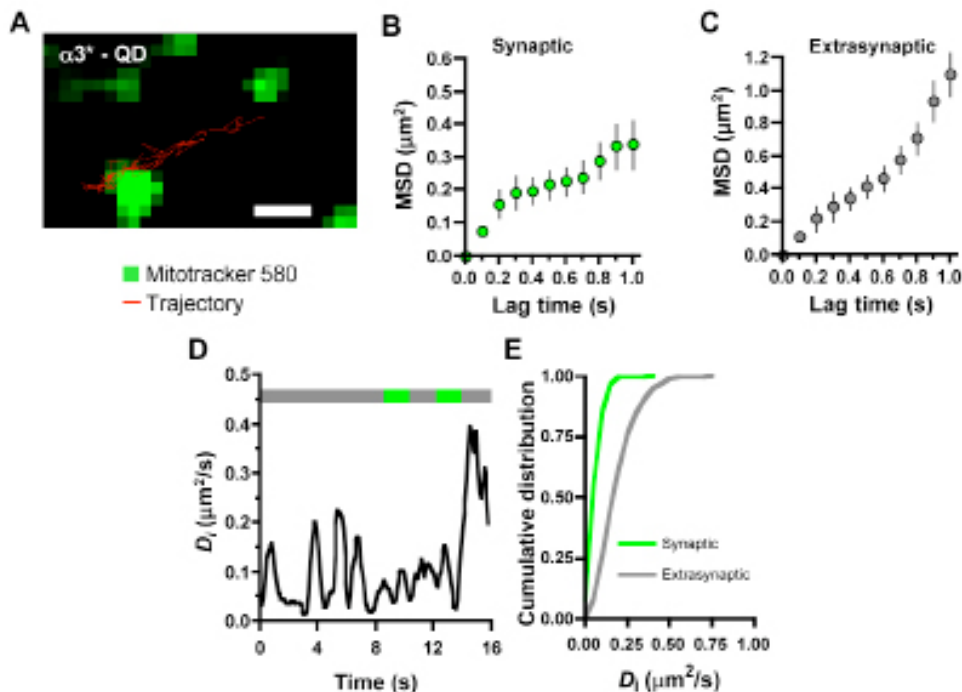
**Figure 3.2 – Expression of  $\alpha 7$ -nAChRs in ciliary ganglion neurons.** Images of  $\alpha 7$ -nAChRs clusters ( $\alpha 7$ ; red in merge) on neurites in juxtaposition to presynaptic structures revealed by the SV2/synaptophysin immunostaining (SV2/Syn; green in merge). Arrows indicate examples of receptor-presynaptic alignment. Scale bar: 5  $\mu$ m (left).

defined by SV2 and synaptophysin immunostaining (Fig 3.2).

### 3.2.2 LATERAL MOBILITY OF nAChRs ON NEURONS

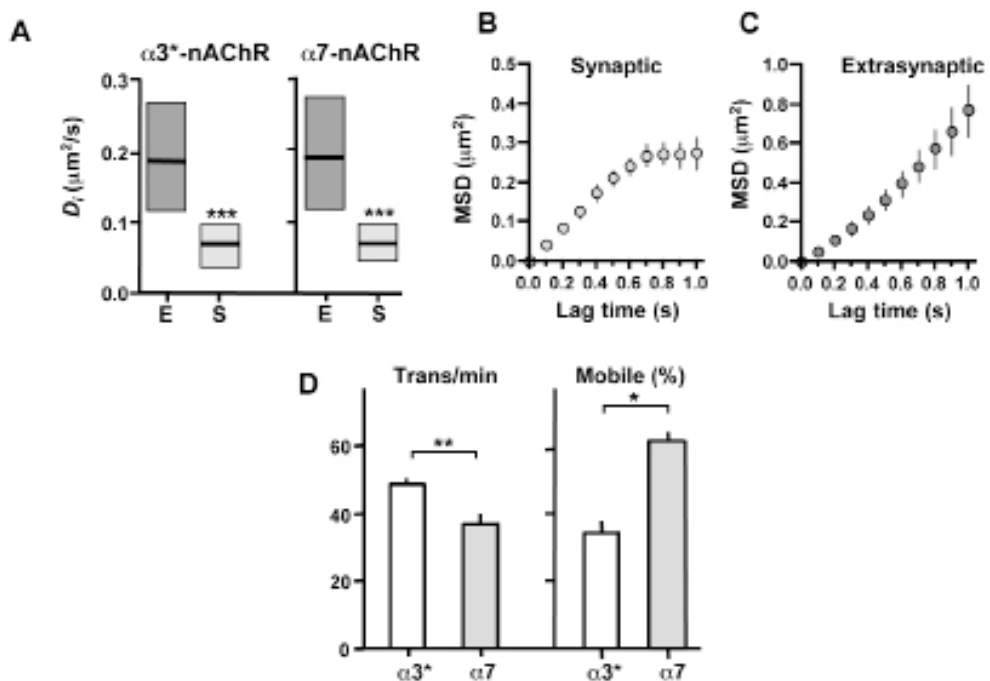
SPT technique with QD nanocrystals (Heine et al., 2008b; Triller and Choquet, 2008) was used

to follow individual nAChRs on the surface of CG neurons in culture. To specifically examine the lateral mobility of nAChRs on neurites of CG neurons in culture,  $\alpha 3^*$ -nAChRs were labeled with Biot-mAb 35 and  $\alpha 7$ -nAChRs with Biot- $\alpha$ -Bgt. In order to reconstruct individual trajectories and to avoid possible particle crosslinking, labelling is usually performed at a low density. QDs linked to streptavidin were tagged through biotinylated ligands and allowed tracking of nAChRs. Synaptic sites were identified for this purpose by pre-labeling either with FM4-64 uptake or MitoTracker staining. Focusing on single nAChR-QD complexes as evidenced by their characteristic blinking, I tracked the movement of  $\alpha 3^*$ -nAChRs that crossed a synaptic/extrasynaptic border at least twice during their trajectory (Fig. 3.3A; Supplemental Movie 1 in <http://www.jneurosci.org/content/30/26/8841/suppl/DC1> ). In SPT experiments, trajectories are recorded and subsequently analyzed individually. The pertinent parameters to be extracted from trajectories are the diffusion coefficient, the confinement, the dwell times and the transition frequency. These elements reflect biological properties of the plasma membrane and of the molecular interactions.



**Figure 3.3 – Different mobilities of surface  $\alpha 3^*$ -nAChRs in synaptic and extrasynaptic spaces.** (A) Trajectory (16 s) of a QD- $\alpha 3^*$ -nAChR (red) along a neurite punctuated with synapses stained with MitoTracker (green). Scale bar: 1  $\mu\text{m}$ . (B, C) MSD as a function of time interval for synaptic and extrasynaptic portions of the trajectories (means  $\pm$  SDs). (D) Instantaneous  $D_i$  values as a function of time along the trajectory. Top line indicates position of QD- $\alpha 3^*$ -nAChR in extrasynaptic (grey) or synaptic (green) domains. (E) Cumulative distribution of the instantaneous diffusion coefficients of synaptic (green) and extrasynaptic (grey)  $\alpha 3^*$ -nAChRs ( $n = 407, 567$  for synaptic and extrasynaptic trajectories, respectively, from 5 separate platings;  $p < 0.001$ , MW test).

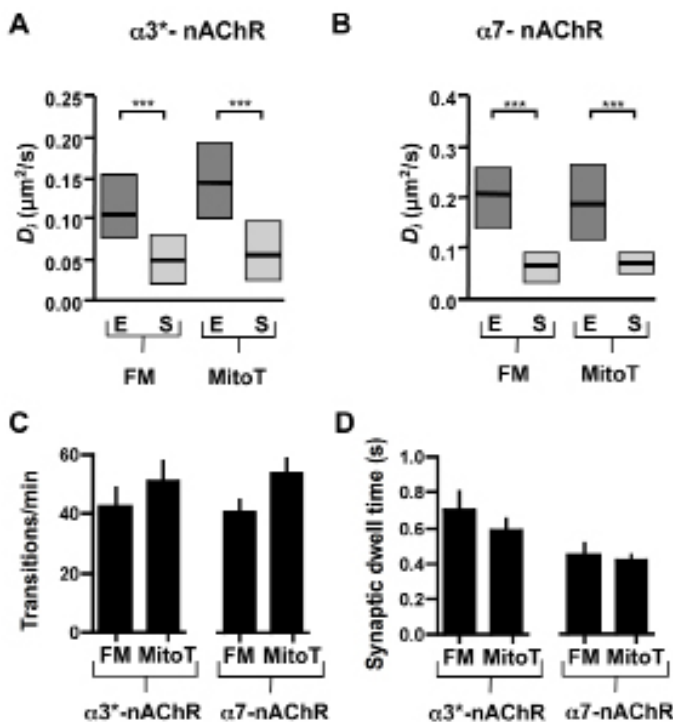
Plots of MSD versus lag time were used to examine mobility quantitatively (Fig. 3.3B,C; see Methods). The initial slope yielded the  $D_i$  for  $\alpha 3^*$ -nAChRs:  $0.070 \mu\text{m}^2/\text{s}$  in synaptic space and  $0.188 \mu\text{m}^2/\text{s}$  in extrasynaptic space (Fig. 3.3D,E;  $p < 0.001$  for synaptic vs. extrasynaptic, MW test). The results were unchanged by a 10-fold reduction in Biot-mAb 35 concentration used to label the receptors, indicating that antibody-receptor cross-linking was unlikely to have skewed the results (not shown). Thus, mobile  $\alpha 3^*$ -nAChRs displayed significantly decreased diffusion rates when in synaptic locations. The linear MSD plot for extrasynaptic space indicated Brownian motion, whereas the non-linear plot in synaptic space is consistent with constrained motion (Kusumi et al., 1993). Remarkably similar results were obtained with QD tracking of mobile  $\alpha 7$ -nAChRs, which showed almost equivalent diffusion rates (Fig. 3.4A; Supplemental movie 2 in <http://www.jneurosci.org/content/30/26/8841/suppl/DC1>). The median  $D_i$  was  $0.067 \mu\text{m}^2/\text{s}$  at synaptic sites and  $0.188 \mu\text{m}^2/\text{s}$  in extrasynaptic regions ( $p < 0.001$ , MW test). MSD plots for a given QD again indicated Brownian motion in extrasynaptic



**Figure 3.4 – Relative mobilities of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs.** (A) Median  $D_i$  values ( $\pm$  25-75% IQR) of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs in synaptic (S) and extrasynaptic (E) space (from 407 S and 567 E trajectories for  $\alpha 3^*$ -nAChRs; 197 S and 312 E for  $\alpha 7$ -nAChRs). (B, C) MSD plots versus time for synaptic and extrasynaptic portions of  $\alpha 7$ -nAChR trajectories (means  $\pm$  SDs). (D) Transition frequency (trans/min) between synaptic and extrasynaptic space for  $\alpha 3^*$ -nAChRs ( $\alpha 3^*$ ) and  $\alpha 7$ -nAChRs ( $\alpha 7$ ;  $n = 86, 57$  QDs) and proportion (mobile %) of mobile  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs ( $n = 124, 245$  total QDs, from 2-5 separate platings). Values in D are means  $\pm$  SEMs.

space and restricted motion in synaptic space (Fig. 3.4B,C). Interestingly,  $\alpha 7$ -nAChRs clearly differed from  $\alpha 3^*$ -nAChRs in the frequency with which they crossed into and out of synaptic space (Fig. 3.4D):  $48.2 \pm 2.5$  and  $36.6 \pm 3.0$  transitions/minute for  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs, respectively ( $p < 0.01$ , t test). They did not show a significant difference in synaptic dwell time (Fig. 2E). Results were the same for synapses marked with MitoTracker staining or with FM4-64 uptake (Fig. 3.5).

Most striking was the difference between  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs on the neurites when immobile receptors were also considered (Fig. 3.4D). Only  $34 \pm 4\%$  of all  $\alpha 3^*$ -nAChRs were mobile compared with  $61 \pm 3\%$  of  $\alpha 7$ -nAChRs ( $p < 0.05$ , t-test). About half of the total immobile receptors, both for  $\alpha 3^*$ -nAChRs and for  $\alpha 7$ -nAChRs, were synaptic (46 and 54%, respectively). Given that synaptic membrane occupies  $6.4 \pm 0.3\%$  of the neurite surface ( $n = 5$  visual fields; neurites visualized by GFP expression and MitoTracker staining, respectively), immobile receptors appear to be preferentially concentrated at synapses ( $\geq 7$ -fold in number per unit area over that in extrasynaptic space). The results indicate that mobile  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs display equivalent diffusion rates in extrasynaptic space and that the synaptic domain restrains their mobility. Additional constraints render a portion of the receptors persistently immobile and collect a significant fraction of such receptors at synapses. This immobility involves



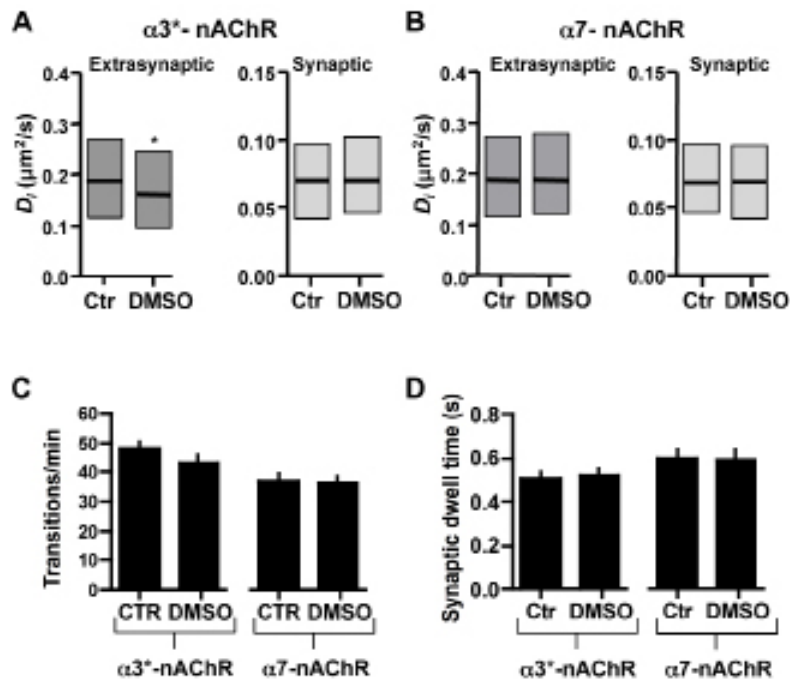
**Figure 3.5 – Comparison of lateral diffusion values for nAChRs using different synaptic markers.** Diffusion properties of receptors were examined in synaptic (S) and extrasynaptic (E) regions on neurites stained either with FM4-64 (5  $\mu M$ , FM) or MitoTracker red 580 (100 nM, MitoT) to label synapses in cultures plated at 1 CG/coverslip. (A) Median  $D_i$  values ( $\pm$  25-75% interquartile range, IQR) for  $\alpha 3^*$ -nAChRs ( $n = 30$ -62 trajectories). (B) Values for  $\alpha 7$ -nAChRs ( $n = 18$ -120 trajectories). (C) Transition frequencies for  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs between extrasynaptic and synaptic spaces. (D) Dwell times in synaptic space for  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs. In C,D values represent means  $\pm$  SEMs;  $n = 6$ -25 QDs from 2 separate culture platings. \*\*\* $p < 0.001$ , KW test.

a larger fraction of  $\alpha 3^*$ -nAChRs than  $\alpha 7$ -nAChRs, demonstrating the relevance of subunit composition for nicotinic receptor fate on neurites.

### 3.2.3 CYTOSKELETAL DETERMINANTS OF nAChRs MOBILITY

When expressed in the cell membrane, nAChRs interact with different cellular components, like proteins and lipids. These interactions affect expression, targeting, function, and signaling of nAChRs. Cytoskeletal elements such as microtubules and filamentous actin (F-actin) can influence the mobility of membrane components on the surface of neurites, differentially affecting receptors in synaptic and extrasynaptic domains (Charrier et al., 2006; Renner et al., 2009).

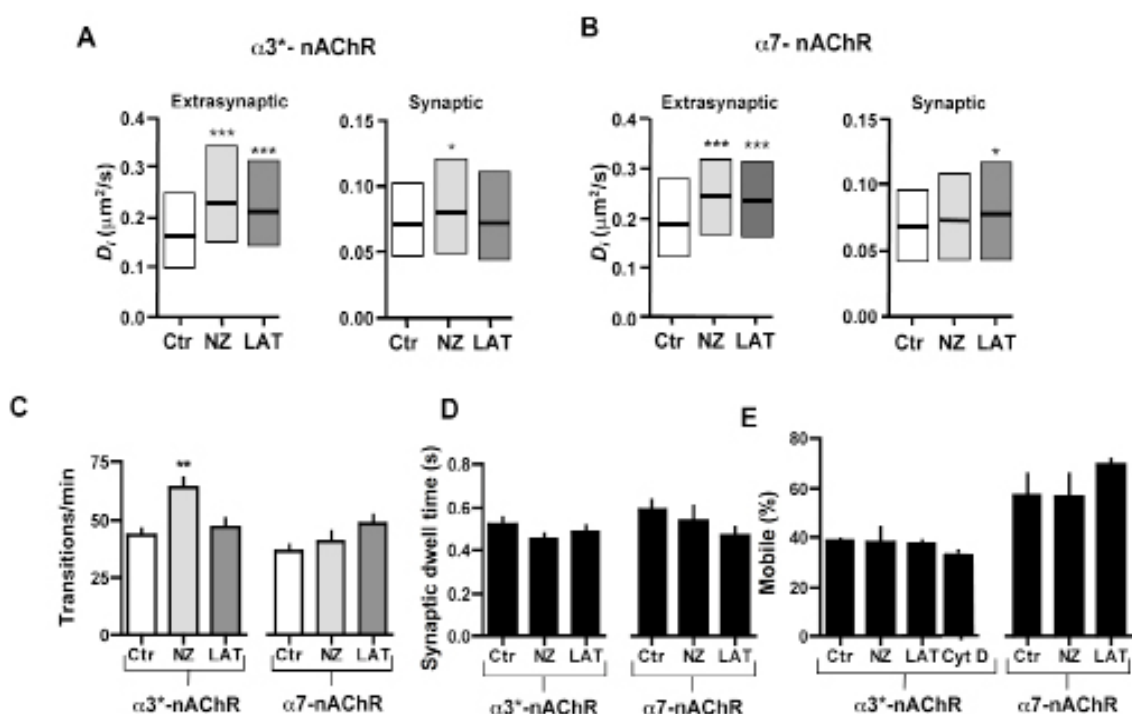
To determine whether microtubules play such a role for nicotinic receptors, incubated CG neurons were incubated with nocodazole (10  $\mu$ M) for 1 hour to depolymerize them; QD trafficking was used to follow  $\alpha 3^*$ -nAChRs on the neurite surface. The vehicle, DMSO, had no effect except for a minor slowing of extrasynaptic  $\alpha 3^*$ -nAChRs (Fig.



**Figure 3.6 – Lateral diffusion of nAChRs in the presence of the vehicle DMSO.** Diffusion properties of receptors were examined in extrasynaptic and synaptic regions on neurites in normal medium (Ctr) or in medium containing 0.1% DMSO. (A) Median  $D_l$  values ( $\pm$  25-75% IQR) for  $\alpha 3^*$ -nAChRs ( $n = 204$ -567 trajectories;  $*p < 0.05$ , MW test). (B) Values for  $\alpha 7$ -nAChRs ( $n = 197$ -381;  $p > 0.05$ , MW test). (C) Transition frequencies between extrasynaptic and synaptic spaces. (D) Dwell times in synaptic space. In C,D values represent means  $\pm$  SEMs ( $n = 24$ -86 QDs;  $p > 0.05$ , t-test; 3-5 separate culture platings).

3.6); all subsequent comparisons were made against vehicle-treated controls.

Nocodazole increased the mobility of the  $\alpha 3^*$ -nAChRs both in synaptic and extrasynaptic space (Fig. 3.7A). The median  $D_i$  values were  $0.079 \mu\text{m}^2/\text{s}$  and  $0.230 \mu\text{m}^2/\text{s}$ , respectively; both were significantly different from those in vehicle conditions ( $p < 0.01$  and  $0.001$ ; KW test). Microtubule depolymerization also increased the frequency of transitions between the two domains (Fig. 3.7C):  $64 \pm 4$  and  $43 \pm 3$  transitions/minute with and without nocodazole treatment, respectively ( $p < 0.01$ ; one-way ANOVA). No significant change was seen in the synaptic dwell time (Fig. 3.7D). Collapse of F-actin by treating with latrunculin A ( $3 \mu\text{M}$ ) for 1 hour also increased the mobility of  $\alpha 3^*$ -nAChRs in extrasynaptic space (Fig. 3.7A). The median  $D_i$  was  $0.210 \mu\text{m}^2/\text{s}$  ( $p < 0.001$  compared to vehicle; KW test). The treatment did not, however, change the fraction of mobile  $\alpha 3^*$ -nAChRs or their synaptic transitions or dwell time (Fig. 3.7A,C,D,E). Both nocodazole



**Figure 3.7 – Different cytoskeletal regulation for  $\alpha 3^*$ -nAChR and  $\alpha 7$ -nAChR lateral diffusion.** QD-nAChR trajectories were examined in extrasynaptic and synaptic space for control conditions (Ctr, 0.1% DMSO) or after disruption of microtubules with nocodazole ( $10 \mu\text{M}$ , NZ) or F-actin with latrunculin A ( $3 \mu\text{M}$ , LAT). (A) Median  $D_i$  values ( $\pm 25$ -75% IQR) for  $\alpha 3^*$ -nAChRs ( $n = 179$ -343 trajectories). (B) Values for  $\alpha 7$ -nAChRs (86-381 trajectories). (C) Transition frequency between synaptic and extrasynaptic space ( $n = 27$ -56 QDs from 2-3 separate platings). (D) Synaptic dwell times of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChR were similar in cultures treated with vehicle (0.1% DMSO, Ctr) and either nocodazole ( $10 \mu\text{M}$ , NZ) to depolymerize microtubules or latrunculin A ( $3 \mu\text{M}$ , LAT) to collapse F-actin ( $n = 27$ -56). (E) The mobile fractions of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChR were not changed by nocodazole, latrunculin A, or cytochalasin D ( $2 \mu\text{M}$ , CytD;  $n = 82$ -203 QDs). Values C, D and E represent means  $\pm$  SEMs; 2-3 separate platings ( $p > 0.05$ , one-way ANOVA).

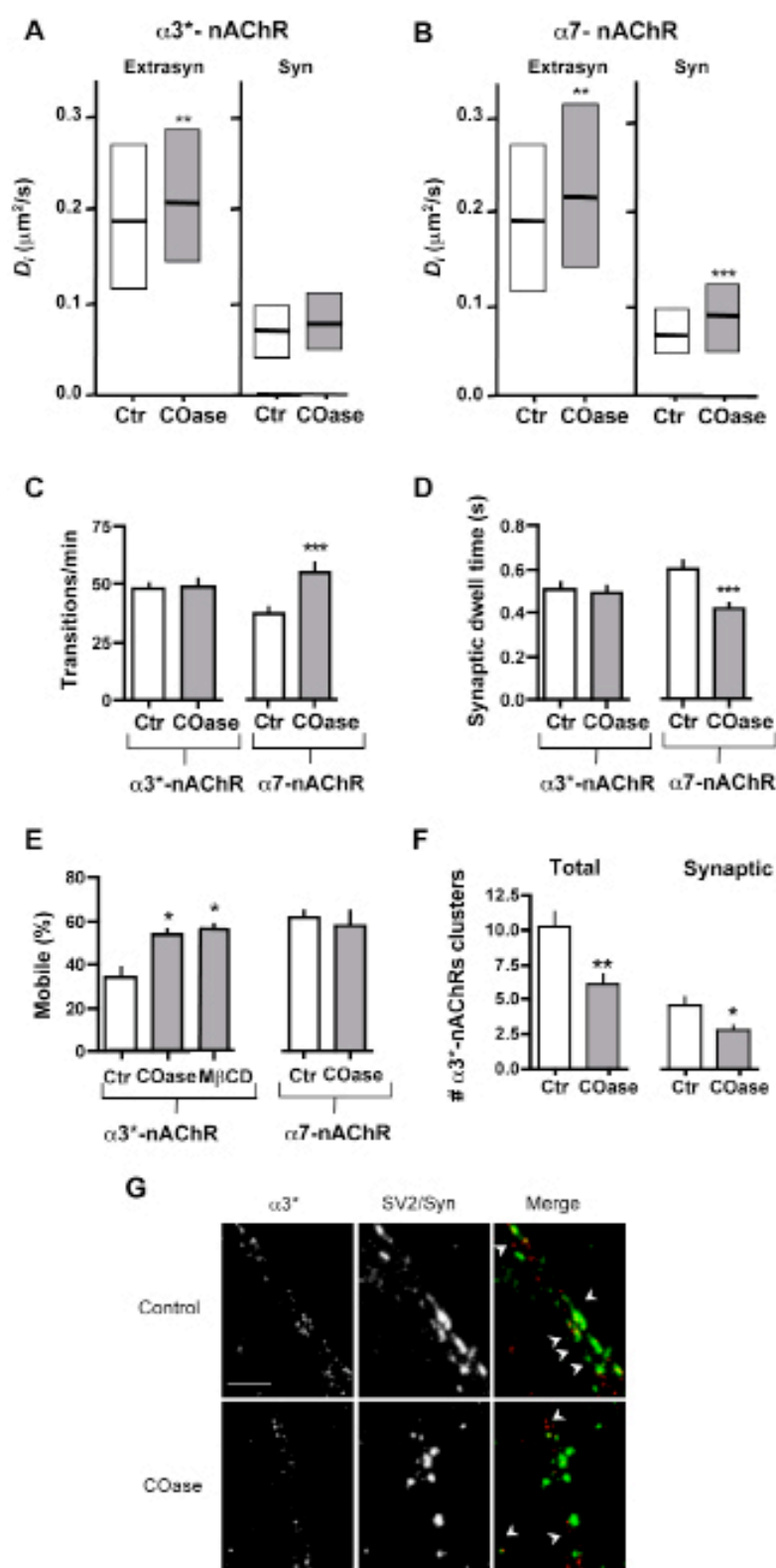
and latrunculin A had different effects on  $\alpha 7$ -nAChRs from those seen for  $\alpha 3^*$ -nAChRs. Nocodazole increased the diffusion of  $\alpha 7$ -nAChRs in extrasynaptic regions; the  $D_i$  was  $0.242 \mu\text{m}^2/\text{s}$  ( $p \leq 0.001$  compared to vehicle; KW test). It had no effect on  $\alpha 7$ -nAChR diffusion inside the synaptic space or on synaptic dwell time or number of transitions (Fig. 3.7C). Collapse of F-actin with latrunculin A increased the mobility of  $\alpha 7$ -nAChRs in both domains (Fig. 3.7B). Median  $D_i$  values of  $0.078 \mu\text{m}^2/\text{s}$  and  $0.233 \mu\text{m}^2/\text{s}$  were obtained for synaptic and extrasynaptic space, respectively ( $p < 0.05$ ,  $p < 0.001$  versus vehicle; KW test). As found for nocodazole, latrunculin A had no effect on the frequency of transitions between the two domains (Fig. 3.7C) or the synaptic dwell time (Fig. 3.7D). Neither nocodazole nor latrunculin A treatment changed the proportions of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs that remained immobile (Fig. 3.7E). Cytochalasin, used as an alternative method of disrupting F-actin, similarly failed to alter the proportion of  $\alpha 3^*$ -nAChRs that were mobile (Fig. 3.7E).

The results indicate that both microtubules and F-actin influence the mobility of nAChRs on neurites but exert different effects depending on synaptic versus extrasynaptic locations and discriminate between  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs. By changing the cytoskeleton's local properties, the effective diffusion constant of nAChRs is modified probably due to reorganization of submembranous obstacles. Neither microtubule nor F-actin constraints, however, can account for the fact that a fraction of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs are immobile, independent of synaptic location.

### 3.2.4 Effects of Cholesterol Depletion on Receptor Mobility

Lipid rafts, which are membrane microdomains rich in cholesterol and glycosphingolipids, can influence the distribution of surface components (Edidin et al., 2003; Marguet et al., 2006; Renner et al., 2009). By governing protein-protein and protein-lipid interactions, these domains selectively incorporate or exclude proteins and therefore have been proposed to function as membrane platforms for the sorting of molecules to particular cellular structures. On CG neurons in vivo, lipid rafts engulf and help stabilize  $\alpha 7$ -nAChRs (Brusés et al., 2001; Liu and Berg, 1999). It has also been suggested that lipid rafts play a role in the stabilization of  $\alpha 3^*$ -nAChRs on the cell membrane (Liu et al., 2008).







Lipid rafts were depleted by incubating CG neurons with COase (2 U/ml), which catalyzes the oxidation of cholesterol to cholestenone and disperses lipid microdomains (Scheiffele et al., 1997; Harder et al., 1998; Keller and Simons, 1998). The treatment increased the mobility of  $\alpha 3^*$ -nAChRs in the extrasynaptic space (median  $D_i = 0.208 \mu\text{m}^2/\text{s}$ ;  $p < 0.01$  compared to vehicle; MW test) but had no effect on synaptic  $\alpha 3^*$ -nAChR mobility, transitioning, or dwell time (Fig. 3.8A,C,D). In contrast, lipid raft disruption had extensive effects on  $\alpha 7$ -nAChRs. Diffusion speeds were increased both within and outside synaptic spaces (Fig. 3.8B). Median  $D_i$  values were  $0.091 \mu\text{m}^2/\text{s}$  and  $0.213 \mu\text{m}^2/\text{s}$  for synaptic and extrasynaptic space, respectively ( $p < 0.001$ ,  $p < 0.01$  compared to vehicle; MW test). In addition,  $\alpha 7$ -nAChRs underwent more transitions into and out of synaptic space (Fig. 3.8C):  $55 \pm 4$  transitions/minute ( $p < 0.001$ ; t test). They also displayed a reduced dwell time at synapses (Fig. 4D):  $0.42 \pm 0.02 \text{ s}$  ( $p < 0.001$ ; t test). Unexpectedly, lipid raft disruption substantially increased the proportion of  $\alpha 3^*$ -nAChRs that displayed mobility (Fig. 3.8E), increasing it from  $34 \pm 4\%$  in controls to  $54 \pm 2\%$  ( $p < 0.05$ ; t test) on neurites of COase-treated cells. A second method of disrupting lipid rafts, namely treating cells with M $\beta$ CD, had the same effect as COase; it increased the proportion of mobile  $\alpha 3^*$ -nAChRs to the same extent. COase treatment had no effect on the proportion of  $\alpha 7$ -nAChRs that were mobile (Fig. 3.8E). Taken together, the results indicate multiple effects of the lipid environment on the mobility of nAChRs. It can immobilize a fraction of receptors depending on their subunit composition (e.g.  $\alpha 3^*$ -nAChRs, but not  $\alpha 7$ -nAChRs) while having little effect on the diffusion of synaptic  $\alpha 3^*$ -nAChRs once mobile. Paradoxically, the lipid raft appears to have greatest effect on the diffusion of  $\alpha 7$ -nAChRs, retarding them both in synaptic and extrasynaptic space, but apparently does not provide the restraint that holds a fraction of  $\alpha 7$ -nAChRs immobile on the surface.

Immobility may be the most relevant criterion for stabilizing receptors at postsynaptic

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**-> Figure 3.8 – Selective effects of cholesterol depletion.** QD-nAChR trajectories were examined in extrasynaptic and synaptic space for control conditions (Ctr) or after cholesterol depletion with either COase or M $\beta$ CD. (A) Median  $D_i$  values for  $\alpha 3^*$ -nAChRs ( $\pm 25\text{-}75\%$  IQR;  $n = 167\text{-}567$  trajectories). (B) Values for  $\alpha 7$ -nAChRs ( $n = 171\text{-}312$ ). (C) Transition frequency ( $n = 38\text{-}86$ ). (D) Synaptic dwell time ( $n = 38\text{-}86$ ). (E) Proportions of mobile  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs ( $n = 124\text{-}245$  total QDs, from 2-5 separate platings). (F) Number of total (left) and synaptic (right)  $\alpha 3^*$ -nAChRs clusters expressed per  $10 \mu\text{m}$  segment of neurite. (G) Arrows indicate examples of alignment for  $\alpha 3^*$ -nAChR clusters ( $\alpha 3^*$ ; red in merge) with presynaptic puncta defined by SV2/synaptophysin immunostaining (SV2/Syn; green in merge). Scale bar:  $5 \mu\text{m}$ . In panels C-F, values represent means  $\pm$  SEMs taken from  $n \geq 3$  separate platings.

sites. The COase treatment, which decreased the number of immobile  $\alpha 3^*$ -nAChRs, also substantially reduced the number of  $\alpha 3^*$ -nAChR clusters on the cell surface (Fig. 3.8 F,G). These included clusters at synaptic sites as defined by proximity to presumptive presynaptic puncta that co-stained for SV2 and synaptophysin (Fig. 3.8F,G). COase produced no decrement in the number of SV2/synaptophysin puncta, arguing against a major presynaptic effect ( $6.2 \pm 0.6$  and  $6.5 \pm 0.5$  for controls and COase, respectively;  $n = 3$  platings). Nor did it reduce the number of  $\alpha 7$ -nAChR puncta ( $6.8 \pm 0.8$  and  $7.6 \pm 0.5$  for controls and COase, respectively;  $n = 3$  platings). No change was seen in the mean size or staining intensity for the remaining puncta in any case. The results are most consistent with the lipid environment providing a restraining component that facilitates immobilization of  $\alpha 3^*$ -nAChRs; release of the constraint tips the balance in favor of dispersal and/or removal.

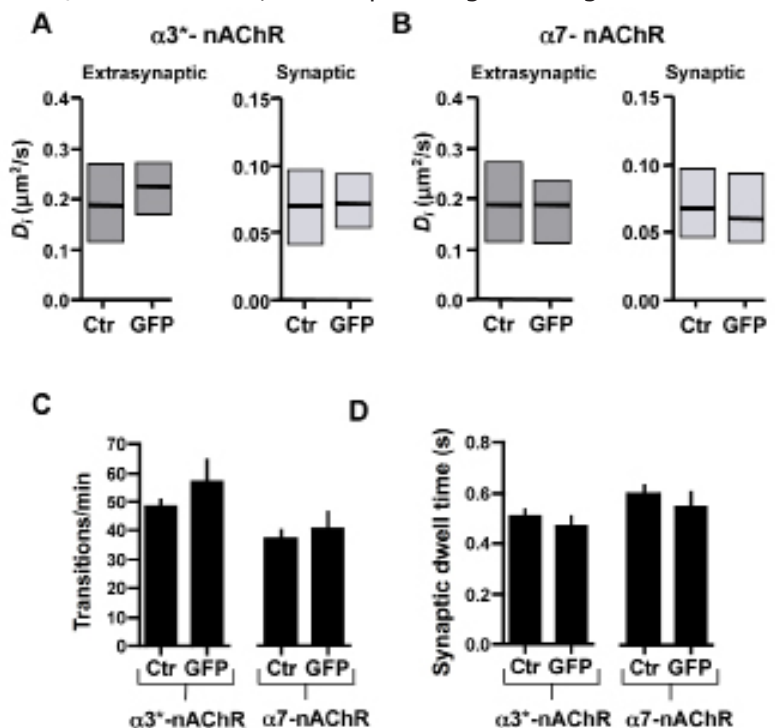
### **3.2.5 CONSTRAINT OF NICOTINIC RECEPTORS BY THE PDZ-CONTAINING POSTSYNAPTIC SCAFFOLDS**

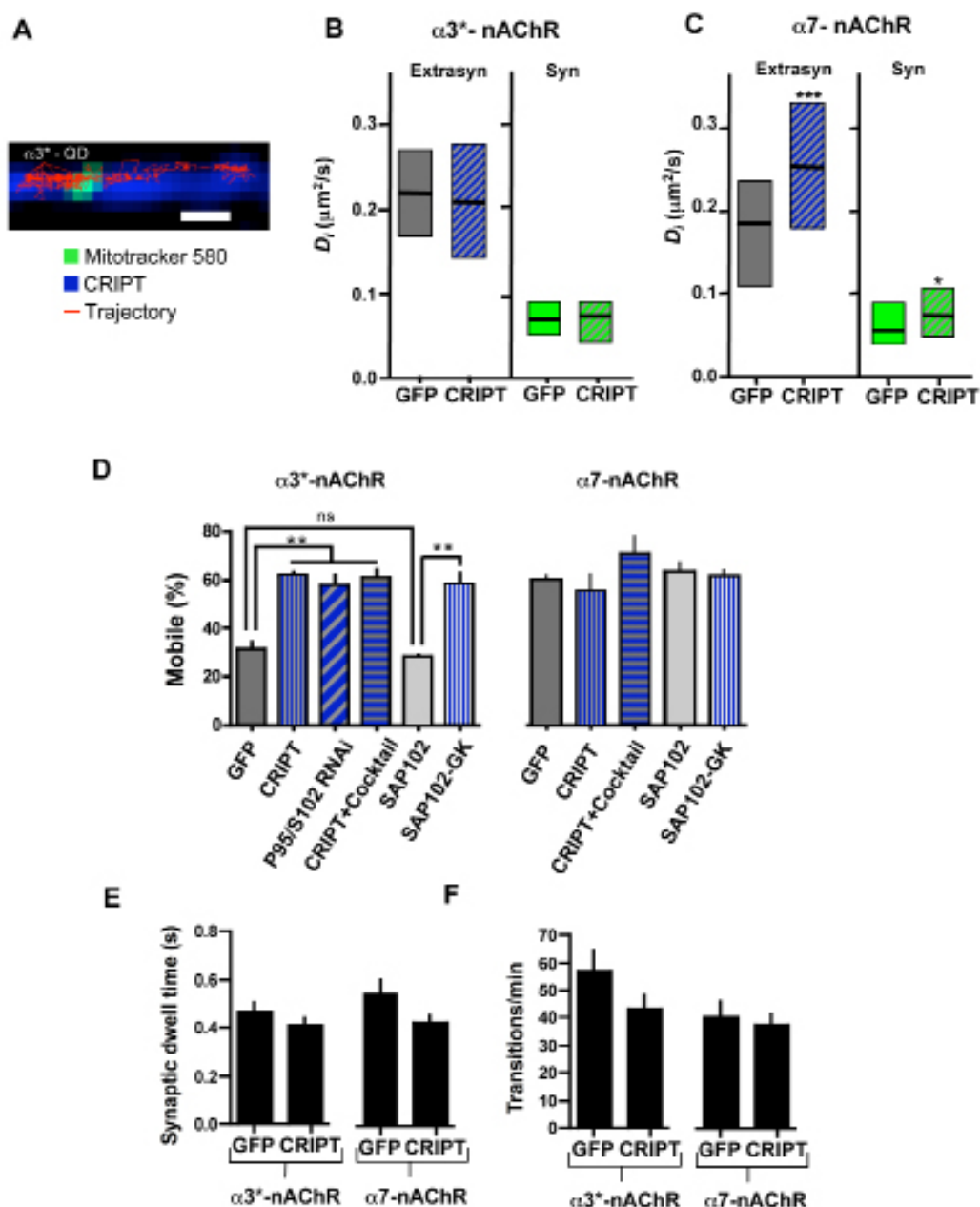
The fact that glutamatergic transmission is responsible for most excitation in the brain, coupled with early difficulties in demonstrating nicotinic postsynaptic potentials in CNS circuits, led to the view that nicotinic receptors might not be found in conventional postsynaptic structures like PSDs.

Members of the PSD-95 family of PDZ-containing proteins establish postsynaptic scaffolds associated with nicotinic receptors on neurons and influence their signaling and localization (Conroy et al., 2003, Parker et al., 2004; Temburni et al., 2004; McCann et al., 2008). To test the extent to which these PDZ-proteins may constrain the lateral mobility of nAChRs, we transfected CG neurons with a construct encoding a 9 amino acid fragment of cysteine-rich interactor of PD 23 (CRIPT) that recognizes the PDZ3 domain of PSD-95 family members and interferes with their ability to form clusters (Passafaro et al., 1999; Conroy et al., 2003). Results were compared both to untransfected controls and to neurons transfected with a construct expressing GFP; no differences were seen between the latter two (Fig. 3.9). CRIPT expression had no effect on the diffusion parameters of mobile  $\alpha 3^*$ -nAChRs (Fig. 3.10A,B) but clearly increased the diffusion of  $\alpha 7$ -nAChRs both in synaptic and extrasynaptic space (Fig. 3.10C; Supplemental Movie 3 in <http://www.jneurosci.org/content/30/26/8841/>

suppl/DC1). Median  $D_i$  values were  $0.078 \mu\text{m}^2/\text{s}$  and  $0.253 \mu\text{m}^2/\text{s}$  for synaptic and extrasynaptic space, respectively ( $p < 0.05$ ,  $p < 0.001$  versus vehicle; MW test). No significant differences were seen for  $\alpha 3^*$ -nAChRs or for  $\alpha 7$ -nAChRs with respect to synaptic dwell time or frequency of transition between synaptic and extrasynaptic space (Fig. 3.10E,F). CRIPT expression, however, doubled the fraction of mobile  $\alpha 3^*$ -nAChRs (Fig. 3.10D), increasing it from  $30 \pm 5\%$  in controls to  $62 \pm 2\%$  ( $p < 0.05$ , t test) in neurites of CRIPT-transfected neurons. To corroborate the results that the target of CRIPT action belongs to the PSD-95 family, neurons were transfected with a RNA interference (RNAi) construct (PSD-95/SAP102-RNAi) that knocked down PSD-95 and SAP102 levels (Neff et al., 2009). These constructs had a similar effect than CRIPT had by itself on the  $\alpha 3^*$ -nAChR mobile fraction (Fig. 3.10D). CRIPT expression produced no change in the fraction of  $\alpha 7$ -nAChRs that was mobile. And no additional receptors were mobilized even when the disruptive treatments were combined: CRIPT transfection for PDZ-scaffolds, nocodazole for microtubules, latrunculin A for F-actin, and COase for lipid rafts. The proportions of mobile  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs were the same as that seen with CRIPT expression alone (Fig. 3.10D). Notably, transfecting cells with a SAP102 construct that lacked a GK domain (SAP102-GK) acted as a dominant negative, replicating the effects of PSD-95/SAP102-RNAi; overexpressing full-length SAP102 had

**Figure 3.9 – Expression of GFP via transfection as a negative control does not change nAChR diffusion properties.** Lateral diffusion of receptors was examined in extrasynaptic and synaptic regions on neurites in untransfected (Ctr) or GFP-transfected (GFP) neurons. (A) Median  $D_i$  values ( $\pm 25$ -75% IQR) for  $\alpha 3^*$ -nAChRs ( $n = 49$ -567;  $*p > 0.05$ , MW test). (B) Values for  $\alpha 7$ -nAChRs ( $n = 66$ -312;  $*p > 0.05$ , MW test). (C) Transition frequencies between extrasynaptic and synaptic spaces. (D) Dwell times in synaptic space. In C,D values represent means  $\pm$  SEMs ( $n = 12$ -86;  $p > 0.05$ , t-test; 3-6 separate culture platings).





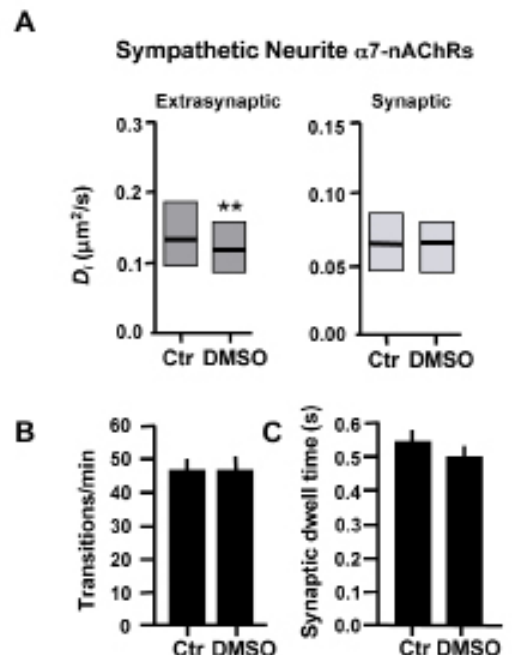
**Figure 3.10 – Regulation of nAChR mobility by PDZ-containing proteins.** (A) QD- $\alpha 3^*$ -nAChR trajectory (red) crossing synaptic spaces labeled with MitoTracker (green) on a CG neurite expressing CRIPT (blue). Scale bar: 1  $\mu\text{m}$ . (B) Median  $D_i$  values ( $\pm$  25-75% IQR) for  $\alpha 3^*$ -nAChRs in extrasynaptic and synaptic space on neurites expressing GFP (control) or CRIPT ( $n = 49$ -66 trajectories). (C) Values for  $\alpha 7$ -nAChRs ( $n = 66$ -145). (D) Proportions of mobile  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs on neurites expressing GFP, CRIPT, PSD-95/SAP102-RNAi (P95/S102), SAP102, or SAP102-GFP (mean  $\pm$  SEM;  $n=40$ -69 total QDs from 2-6 separate platings). Cocktail: treatment with nocodazole (10  $\mu\text{M}$ ), latrunculin A (3  $\mu\text{M}$ ), and COase treatments (2 U/ml). (E) Dwell times in synaptic space. (F) Transition frequencies between extrasynaptic and synaptic spaces. Values in E-F are means  $\pm$  SEMs ( $n = 12$ -16;  $p > 0.05$ , t-test; 3-6 separate platings).

no effect (Fig. 3.10D). None of the treatments produced a change in the fraction of  $\alpha 7$ -nAChRs that was mobile. The results show that PDZ-containing proteins differentially affect the mobilities of  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs, effectively discriminating on the basis of subunit composition. They constrain the rates of diffusion for mobile  $\alpha 7$ -nAChRs but not for mobile  $\alpha 3^*$ -nAChRs, and conversely, they increase the size of the  $\alpha 3^*$ -nAChR mobile fraction via a GK domain without affecting this parameter for  $\alpha 7$ -nAChRs.

### 3.2.6 MOBILITY OF $\alpha 7$ -nAChRs ON CHICK SYMPATHETIC GANGLION NEURONS

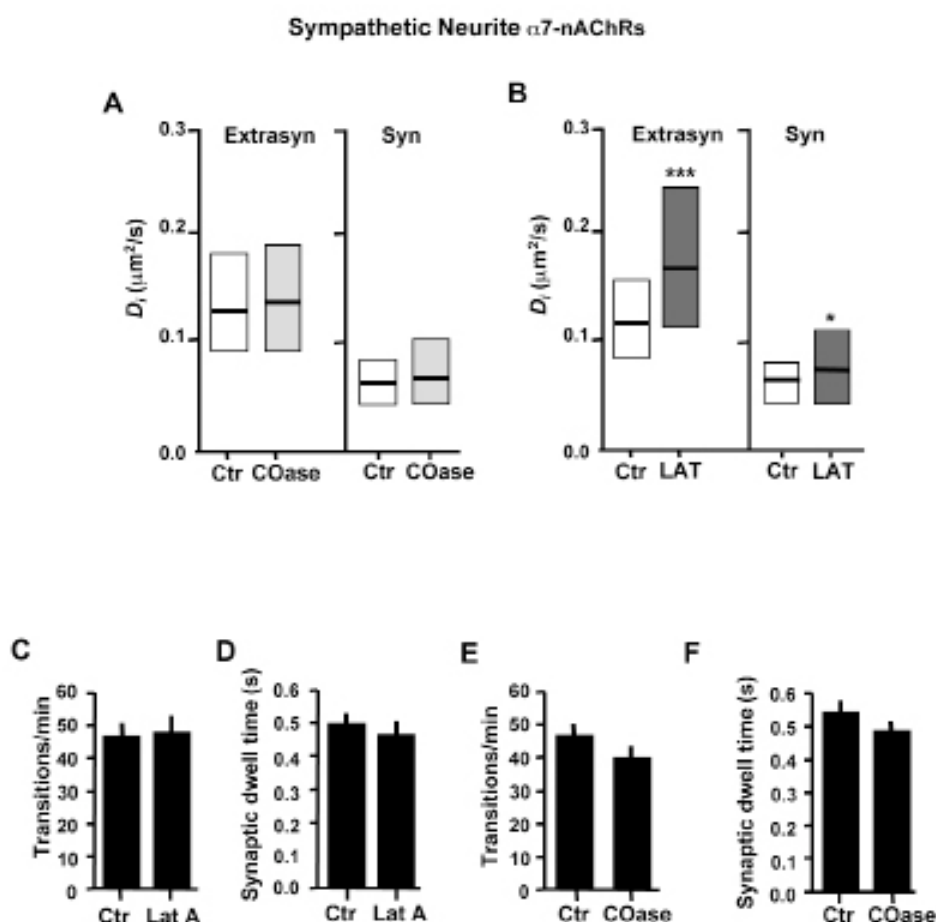
To assess the generality of these results, experiments were also carried out with chick sympathetic ganglion neurons in culture.

The neurons express  $\alpha 7$ -nAChRs that can be tagged with Biot- $\alpha$ Bgt and QDs and followed through synaptic and extrasynaptic spaces on neurites. Analyzing the trajectories and calculating the  $D_i$  values as described above for CG neurons indicated again a restricted mobility for  $\alpha 7$ -nAChRs in synaptic space, compared to extrasynaptic space (Fig. 3.11A). The synaptic  $D_i$  was equivalent on sympathetic and CG neurites, but the extrasynaptic  $D_i$  was different ( $p < 0.001$ , MW test). Disrupting the actin cytoskeleton with latrunculin A produced increases in the diffusion rates of  $\alpha 7$ -nAChRs both in synaptic and extrasynaptic space (Fig. 3.12B), and the increases were comparable to those seen for  $\alpha 7$ -nAChRs on CG neurons. A clear difference, however, emerged when lipid rafts were disrupted. Unlike the effect on CG neurons, treating sympathetic ganglion neurons with COase produced no change in the



**Figure 3.11 – Effect of vehicle (DMSO) on the lateral diffusion of  $\alpha 7$ -nAChRs on sympathetic neurites.** Diffusion properties of receptors were examined in extrasynaptic and synaptic regions on neurites in normal medium (Ctr) or in medium containing 0.1% DMSO. (A) Median  $D_i$  values ( $\pm 25$ -75% IQR) for  $\alpha 7$ -nAChRs ( $n = 121$ -264; \*\* $p < 0.01$ , MW test). (B) Transition frequencies between extrasynaptic and synaptic spaces. (C) Dwell times in synaptic space. In B, C values represent means  $\pm$  SEMs ( $n = 30$ -39;  $p > 0.05$ , t-test; 3 separate culture platings).

mobility of  $\alpha 7$ -nAChRs either in synaptic or extrasynaptic space (Fig. 3.12A), raising the possibility that there might be regional variation in raft-receptor interactions. The results indicate that the general features of  $\alpha 7$ -nAChR mobility are common among the two cell types but that regulatory controls can also be cell type-specific.



**Figure 3.12 – Lateral diffusion of surface  $\alpha 7$ -nAChRs in synaptic and extrasynaptic space on sympathetic ganglion neurites.** (A) Median  $D_i$  values ( $\pm$  25-75% IQR) for extrasynaptic ( $n = 193$ -264) and synaptic ( $n = 91$ -196) trajectories in control conditions (Ctr, 0.1% DMSO) or after cholesterol depletion with cholesterol oxidase (2 U/ml, COase). (B) Median  $D_i$  values ( $\pm$  25-75% IQR) for extrasynaptic ( $n = 170$ -207) and synaptic ( $n = 102$ -121) trajectories in control conditions (Ctr, 0.1% DMSO) or after disruption of F-actin with latrunculin A (3  $\mu\text{M}$ , LAT). Transition frequencies (C) between extrasynaptic and synaptic spaces and synaptic dwell times (D) were calculated for control conditions (Ctr, 0.1% DMSO) or after disruption of F-actin with latrunculin A (3  $\mu\text{M}$ , LAT). Transition frequencies (E) between extrasynaptic and synaptic spaces and synaptic dwell times (F) were calculated for control conditions and after cholesterol depletion with COase (2 U/ml). Values in C-F are means  $\pm$  SEMs ( $n = 27$ -39;  $p > 0.05$ , t-test; 3 separate platings).

### 3.3 Discussion

Single-particle-tracking of nAChRs was used to examine the mobility of  $\alpha 7$ - and  $\alpha 3^*$ -nAChRs on the neuron surface. It was found that substantial fractions of both classes of nAChRs are mobile on the surface, that they transition between synaptic and extrasynaptic domains, and that they slow down when transiting synaptic space. In addition, a fraction of each nAChR type is immobile and preferentially localized in synaptic space. Strikingly, most of the  $\alpha 3^*$ -nAChRs appeared to be immobile on the surface of the neuritis, contrasting with  $\alpha 7$ -nAChRs, which were preferentially moving. Disruption of the cytoskeleton, lipid rafts or postsynaptic scaffolds differentially affects  $\alpha 7$ - and  $\alpha 3^*$ -nAChRs. Curiously, the mechanisms of  $\alpha 7$ -nAChR restraint depended, to a certain extent, to the cell type used. Together, these data suggests that different mechanisms constrain receptor populations depending on local domain, receptor subunit composition and cell type.

#### 3.3.1 Mechanisms that control the lateral diffusion of $\alpha 7$ - and $\alpha 3^*$ -nAChRs

The postsynaptic membrane complex in glutamatergic synapses contains transmembrane components that restrict diffusion, as well as scaffold networks that anchor receptors (Kim and Sheng, 2004; Charrier et al., 2006; Renner et al., 2009). The latter includes PSD-95 family members, which have been shown to regulate AMPA receptor mobility (Bats et al., 2007). The lipid environment also restricts mobility by increasing local membrane viscosity (Marguet et al., 2006; Allen et al., 2007) and by concentrating components that tether receptors, such as palmitoylated PSD-95 and the sequestration of AMPA receptors (El-Husseini et al., 2002). Links to the cytoskeleton help secure the postsynaptic membrane complex (Feng and Zhang, 2009).

In this study, I investigated some mechanisms that seemed to be good candidates for constraining nAChRs mobility in neurons. Neither actin filaments nor microtubules changed the proportions of mobile  $\alpha 7$ - and  $\alpha 3^*$ -nAChRs. However, cholesterol depletion by COase (or M $\beta$ CD) or disruption of postsynaptic PDZ-scaffolds by CRIPT (or PSD-95/SAP102 RNAi) selectively freed up a portion of immobile  $\alpha 3^*$ -nAChRs but



not immobile  $\alpha 7$ -nAChRs on CG neurons. Curiously, the fraction of  $\alpha 3^*$ -nAChRs that remained immobile on neurites after CRIPT or COase treatment was comparable in size to the fraction of immobile  $\alpha 7$ -nAChRs found under all conditions. Disruption of postsynaptic PDZ-scaffolds had no effect on the lateral diffusion of already mobile  $\alpha 3^*$ -nAChRs. Paradoxically, it did increase the diffusion of  $\alpha 7$ -nAChRs without changing the proportion of  $\alpha 7$ -nAChRs that was mobile. Combining a cocktail of disrupters with the CRIPT expression (to acutely disrupt microtubules, F-actin, and lipid rafts in addition to chronic disruption of PDZ-links) produced no further decrease in the fraction of immobile receptors. CRIPT and COase treatments may have generated equivalent outcomes because they targeted a shared mechanism, like for example the clustering of palmitoylated PDZ-proteins in a lipid raft (El-Husseini et al., 2002). Other cholesterol-protein interactions, however, may contribute to nAChR constraint (Gimpl et al., 2002; Allen et al., 2007). The TM4 of nAChR subunit contains a highly conserved cysteine residue that appears to be involved in receptor aggregation and interaction with cholesterol and other lipid-related molecules. Cholesterol depletion is also known to increase the activity of several staurosporine-sensitive kinases including PKA, PKC and Src (Burgos et al., 2004; Cabrera-Poch et al., 2004), which could eventually modify the phosphorylation and the function of scaffolds and/or receptors.

The fact that the SAP102-GK construct acted as a dominant negative provided evidence that the PDZ-scaffold constrains receptor mobility by linking them to sites via a GK domain. It has recently been shown that the GK domain of SAP102 is, in fact, responsible for stabilizing the protein at the postsynaptic density (Zheng et al., 2010). The receptor-specific effects caused by disrupting PDZ-scaffolds may be determined by the individual PSD-95 family members that interact with  $\alpha 3^*$ -nAChRs versus  $\alpha 7$ -nAChRs, conferring different types of constraint (Conroy et al., 2003).

Although the relationship between stabilization and aggregation of receptors is not totally clear yet, we found here that molecules that contribute for  $\alpha 3^*$ -nAChRs immobilization seem to also contribute for the aggregation of  $\alpha 3^*$ -nAChRs in clusters. Cholesterol depletion not only freed up immobile  $\alpha 3^*$ -nAChRs, but also decrease the size of  $\alpha 3^*$ -nAChR clusters. The double knockdown of PSD95/SAP102 had the same effect on aggregation/stability of  $\alpha 3^*$ -nAChRs (Neff et al., 2009b and present work). Disruption of PDZ-scaffolds also interferes with signal transduction and downstream



signaling by nAChRs, indicating the importance of receptor positioning (Conroy et al., 2003). Further, PSD-95/SAP102 RNAi decreases paired-pulse depression at synapses on CG neurons (Neff et al., 2009). Some of this latter effect may reflect increased receptor mobility providing rapid receptor exchange that diminishes the effects of desensitization, a phenomenon reported for AMPA receptors (Heine et al., 2008b; Frischknecht et al., 2009). Lateral mobility would appear to be important for recruiting nAChRs to desired locations if receptor localization can then be stabilized.

Changes in receptor diffusion did not necessarily induce a measurable change in either synaptic dwell time or frequency of transitions. This was true for diffusion of mobile  $\alpha 7$ -nAChRs on CG neurons following CRIPT disruption of PDZ-scaffolds. Similarly, F-actin collapse by latrunculin A and microtubules disruption by nocodazole increased diffusion of mobile  $\alpha 7$ -nAChRs and  $\alpha 3^*$ -nAChR, respectively, without changing either their synaptic dwell time or transition frequency. The results suggest that synaptic retention of mobile receptors may not simply reflect slower movement. Instead the receptors may be physically constrained either by a molecular border around the space or by a constraint that tethers them within the space (O'Connell et al., 2006; Triller and Choquet, 2008).

The tracking of endogenously expressed receptors currently requires the use of ligands that bind the receptors of interest (mAb35 and  $\alpha$ -Bgt in our case). It is possible that the binding of ligands can induce conformational changes (Mitra et al., 2001) in the receptors, which could eventually lead to changes in their diffusion. For tracking  $\alpha 7$ -nAChRs, we used the antagonist  $\alpha$ -Bgt due to the inexistence of antibodies that specifically bind the extracellular domain of  $\alpha 7$  subunits. In this case, the inactivation of the receptors by  $\alpha$ -Bgt could greatly affect the diffusion of receptors in the lateral membrane and even change the way that receptors are inserted into and/or removed from synapses. In the future, a good way to control how  $\alpha$ -Bgt interferes with the diffusion of  $\alpha 7$ -nAChRs is doing FRAP experiments with  $\alpha 7$ -nAChRs-GFP, for instance, in the presence and in the absence of the toxin.

### **3.3.2 Context of nAChRs mobility with respect of other transmitter receptors**

The surface diffusion of various neurotransmitter receptors is heterogeneous, with

relatively 'slow' diffusing receptors (e.g., NMDARs) compared to 'faster' ones (e.g., AMPAR). The first report of neurotransmitter receptor lateral diffusion revealed nAChR surface trafficking at the surface of cultured muscle fibers. It was shown that approximately 50% of  $\alpha$ -Bgt sensitive AChRs are mobile, and the mobile fraction diffuse on average at 0.1-0.01  $\mu\text{m}^2/\text{s}$ . Values with the same order of magnitude were found for neuronal nAChRs in the present study. Moreover, the diffusion rates found here for mobile  $\alpha 7$ - and  $\alpha 3^*$ -nAChRs fall within the range reported previously for membrane components traversing synapses (Groc and Choquet, 2008). The 3-fold difference in synaptic vs. extrasynaptic diffusion is comparable to that reported both for AMPA and NMDA receptors (Ehlers et al., 2007). This type of restriction has usually been attributed to limitation of receptor movements by barriers acting as fences (Jacobson et al., 1995; Kusumi and Sako, 1996), by obstacles in the membrane (Daumas et al., 2003), by transient association with specialized lipid microdomains (Dietrich et al., 2002; Anderson and Jacobson, 2002) or by the transient association with receptor-scaffold clusters (Meier et al., 2001; Sergé et al., 2002). It should be noted that membrane-anchored receptors by themselves slow down the particles inside synapses.

In the present study, the diffusion of  $\alpha 7$ -nAChRs was studied in sympathetic and parasympathetic neurons from the PNS. We found that the regulatory controls of  $\alpha 7$ -nAChRs mobility were cell type-specific, since different mechanisms controlled the diffusion  $\alpha 7$ -nAChR in symapathethic and parasympathetic neurons. In the CNS, multiple kinds of nAChRs exist, and they exert numerous effects depending on location and synaptic target (Picciotto et al., 1995; Newhouse et al., 1997; Bannon et al., 1998; Marubio et al., 1999; Mansvelder and McGehee, 2002; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002; Maskos et al., 2005; Bitner et al., 2007; Teper et al., 2007). If the mechanisms that control the mobility of nAChRs are shared by peripheral and central neurons is still a matter to be studied. In addition, the surface diffusion of nAChRs might depend on the developmental stage of the neurons. For example, the lipid composition of neuronal membranes is altered due to cellular maturation and different scaffolds are expressed during development. These changes could lead to alterations in nAChR dynamic as neurons develop and mature.

### 3.3.3 nAChRs – a heterogeneous population of receptors?

There are several possibilities to explain why  $\alpha 3^*$ -nAChRs did show different behaviors regarding their mobility, i.e., why a population of  $\alpha 3^*$ -nAChRs was sensitive to PSD95/SAP102 disruption and other was not. First, on CG neurons,  $\alpha 3^*$ -nAChRs are heterogeneous with respect to their subunit composition. All contain  $\alpha 3$  and  $\beta 4$  subunits, but only some contain  $\beta 2$  subunits and some, but perhaps not all, contain  $\alpha 5$  subunits (Conroy and Berg, 1995). This heterogeneity may explain, in part, why  $\alpha 3^*$ -nAChRs did not respond as a homogeneous population. Even  $\alpha 7$ -nAChRs, which were thought to be exclusively homopentameric (Drisdell and Green, 2000), may in some cases be heteropentameric (Khiroug et al., 2002; Liu et al., 2009). Alternatively, the finding that not all receptors of a given class appear to be constrained by the same component may result from the restraining component being present in limited amounts.

In this study, I found that the lateral diffusion of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs is controlled by different mechanisms. The  $\alpha 3$  and  $\alpha 7$  subunits are highly homologous, but show a major divergence in both sequence and length of the large cytoplasmic loop (Williams et al., 1998). Interestingly, since N- and C-terminals are located in the external part of the cell membrane, the large cytoplasmic loop has been considered the major candidate to interact with cytoplasmic transport machinery for trafficking nAChRs to synapses. Interestingly, it was shown that it is actually the  $\alpha 3$  cytoplasmic loop that targets nAChRs at the synapses (Williams et al., 1998). It would be interesting to follow the dynamic of the chimeric  $\alpha 7$  subunits with the long cytoplasmic loop of  $\alpha 3$  (and of the chimeric  $\alpha 3$  subunits with the long cytoplasmic loop of  $\alpha 7$ ) to study the importance of the long cytoplasmic loop in the lateral diffusion of nAChRs. In addition, the heterogeneity found on the dynamic of nAChRs may be due to posttranslational modifications of the large cytoplasmic loop that could change its structure, allowing the same nAChR subunit to interact with different organizing elements.

### 3.3.4 Functional consequences of nAChR lateral diffusion

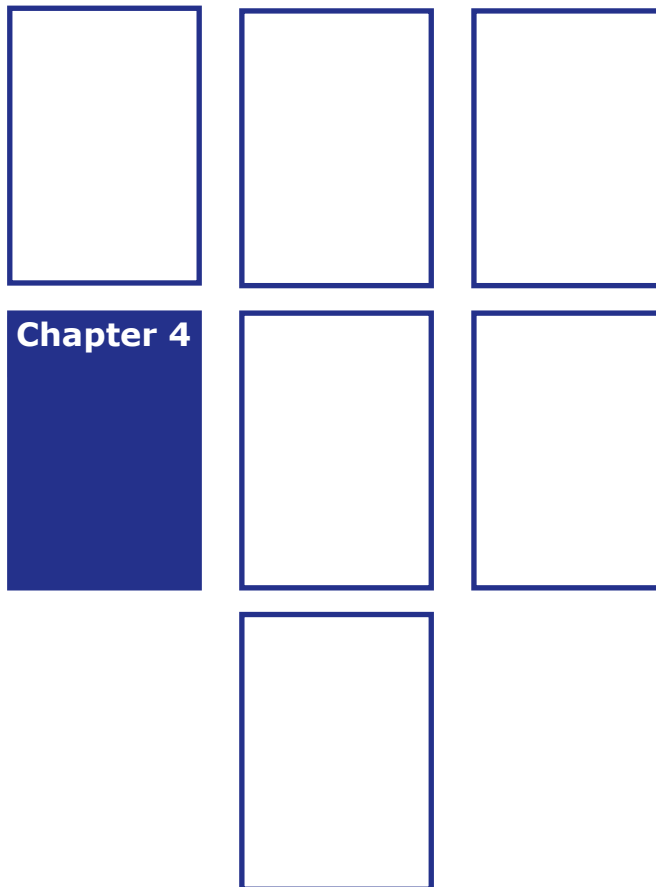
Lateral mobility allows transmitter receptors to be available for quick recruitment at synapses. Our results suggest that nAChRs subunit-specific interactions with

cytoskeletal and scaffold components are likely to be key determinants in constraining otherwise mobile nAChRs to unique locations according to individual receptor subtype and cell identity. This, in turn, can provide a mechanism for targeting individual nAChR subtypes to specific job assignments. Most of the  $\alpha 3^*$ -nAChRs appeared to be immobile on the surface of the neuritis, contrasting with  $\alpha 7$ -nAChRs, which were preferentially moving. The stability of  $\alpha 3^*$ -nAChRs, in comparison with  $\alpha 7$ -nAChRs, might be related with the fact that  $\alpha 3^*$ -nAChRs generate most of the synaptic response in culture (Chen et al., 2001; Conroy et al., 2003).

In this chapter, I examined receptor trafficking on CG neurites that are innervated in culture, as are CG projections at comparable early stages in vivo (Landmesser and Pilar, 1972; Liu et al., 2006). On mature CG neurons in vivo,  $\alpha 3^*$ -nAChRs, but not  $\alpha 7$ -nAChRs, are concentrated at postsynaptic densities. In CG cultures, both  $\alpha 3^*$ -nAChRs and  $\alpha 7$ -nAChRs traverse synaptic space on neurites, but the "synaptic"  $\alpha 7$ -nAChRs seen here could be perisynaptic at the ultrastructural level.

Mechanisms shown here to constrain receptor mobility have been shown to stabilize receptor clusters. Disruption of lipid rafts and collapse of F-actin, for example, each disperses the large  $\alpha 7$ -nAChR clusters found on freshly dissociated CG neuron cell bodies (Shoop et al., 2000; Brusés et al., 2001; Liu and Berg, 1999). Disruption of PSD-95 family PDZ-scaffolds in CG neurons via CRIPT expression or PSD-95/SAP102 RNAi significantly decreases synaptic  $\alpha 3^*$ -nAChRs in culture (Neff et al., 2009).

In the CNS, multiple kinds of nAChRs exist and they exert numerous effects depending on location and synaptic target (Picciotto et al., 1995; Newhouse et al., 1997; Bannon et al., 1998; Marubio et al., 1999; Mansvelder and McGehee, 2002; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002; Maskos et al., 2005; Bitner et al., 2007; Teper et al., 2007). Lateral mobility allows nAChRs to be available for quick recruitment. The results described in this chapter suggest that subunit-specific interactions with cytoskeletal and scaffold components are likely to be key determinants in constraining otherwise mobile nAChRs to unique locations according to individual receptor subtype and cell identity.



**POSTSYNAPTIC ACTION OF BRAIN-DERIVED NEUROTROPHIC  
FACTOR ATTENUATES  $\alpha 7$  NICOTINIC ACETYLCHOLINE RECEPTOR-  
MEDIATED RESPONSES IN HIPPOCAMPAL INTERNEURONS**



## POSTSYNAPTIC ACTION OF BRAIN-DERIVED NEUROTROPHIC FACTOR ATTENUATES $\alpha 7$ NICOTINIC ACETYLCHOLINE RECEPTOR-MEDIATED RESPONSES IN HIPPOCAMPAL INTERNEURONS

### 4.1 INTRODUCTION

Nicotinic signaling influences attention, learning and memory in the hippocampus. (Romanelli et al., 2007). Understanding how nicotinic inputs are modulated is thus expected to reveal new aspects of learning and memory formation. The most likely targets of the cholinergic septohippocampal pathway are the GABAergic interneurons in the hippocampus (Léránth and Frotscher, 1987). These cells highly express diverse subtypes of somato-dendritic nAChRs, including fast desensitizing  $\alpha 7$ -containing receptors, as well as a variety of non- $\alpha 7$  nAChRs (Albuquerque et al., 2009). Although fewer inhibitory neurons and synapses are present in the hippocampus, inhibitory neurons manifest in a bewildering diversity, and their synapses exert a profound influence on the properties of neural circuits (e.g., see Klausberger and Somogyi, 2008). In addition, nAChRs are also located in pyramidal cells, although at a lower level of expression.

The preferential location of nAChRs in interneurons explains, in part, the profound impact of cholinergic transmission on the regulation of synaptic transmission and plasticity in the hippocampus. Activation of  $\alpha 7$ -nAChRs in a single interneuron has the ability to rapidly counteract short- and long-term plasticity phenomena that takes place in the CA1 pyramidal cells (Ji et al., 2001). Interestingly, activation of  $\alpha 7$ -nAChRs expressed on pyramidal cells boosts STP and LTP (Ji et al., 2001). Since temporal and spatial coordination is so important for setting different  $\alpha 7$ -nAChRs-mediated actions, it is crucial to understand the mechanisms that regulate their function. Furthermore, activation of  $\alpha 7$ -nAChRs supplies ionic signals, including  $\text{Ca}^{2+}$  (Bertrand et al., 1993; Seguela et al., 1993), that can eventually culminate in alterations of gene transcription (Chalazonitis and Zigmond, 1980; Greenberg et al., 1986; Pelto-Huikko et al., 1995; Salminen et al., 1999; Gueorguiev et al., 2000; Chang and Berg, 2001).

Knowledge on the first messengers that regulate nAChRs in the hippocampus is still limited. It has been shown that  $\alpha 7$ -nAChR levels on hippocampal neurons in culture can be upregulated by the neurotrophin-gene family member BDNF (Kawai et al., 2002; Massey et al., 2006). These studies showed that BDNF specifically increases  $\alpha 7$ -nAChR number and clustering over a time course of several hours to days in interneurons, although it did not affect  $\alpha 7$ -nAChRs expressed in pyramidal cells. Furthermore, the action of BDNF was dependent on the activation of Trk tyrosine kinase receptors, which are known to engage multiple second messenger pathways (Massey et al., 2006).

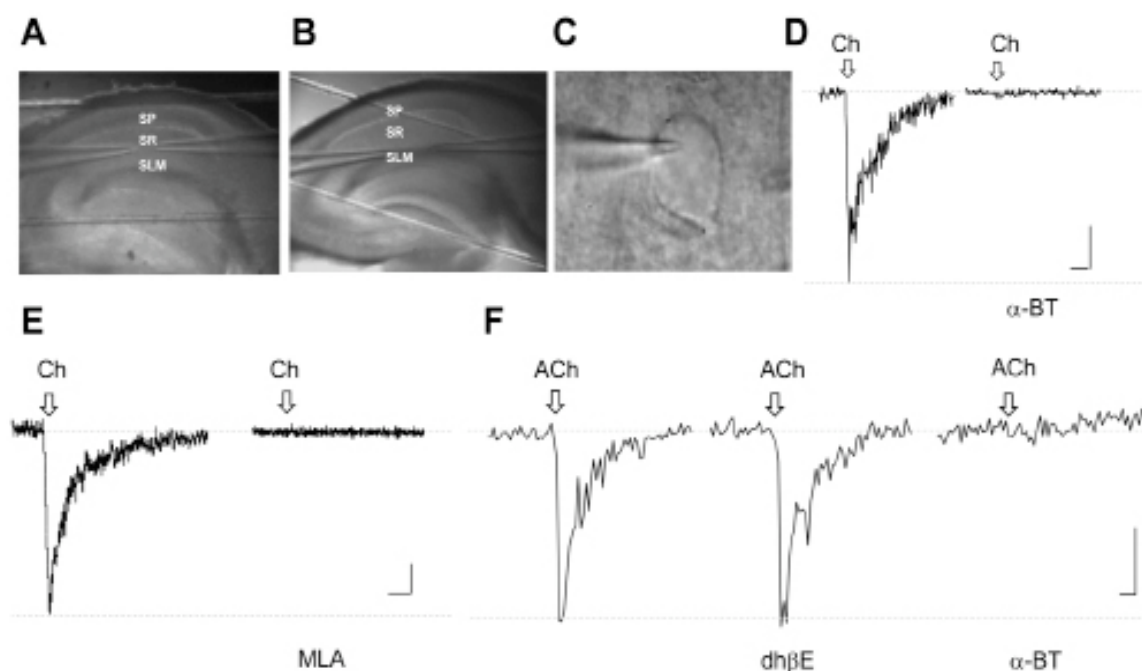
Neurotrophins have been considered regulatory molecules linking rapid changes at synapses with longer-lasting modifications of circuit activity (Poo, 2001). In the last fifteen years, several studies described that short-term regulatory effects precedes BDNFs' long-term regulation on the expression of transmitter receptors. For instance, BDNF was found to modify the properties of postsynaptic transmitter channels, including AMPA, NMDA, and GABA<sub>A</sub> receptors in short- and long-term scales (Rose et al., 2004). In this chapter, **I investigated whether BDNF drives rapid changes in the activity of  $\alpha 7$  nACh receptors in inhibitory cells of the hippocampus and examined the downstream effectors involved in that putative crosstalk.**

## 4.2 RESULTS

### 4.2.1 BDNF INDUCES A RAPID DEPRESSION OF $\alpha 7$ -nAChR-MEDIATED CURRENTS

The activity of  $\alpha 7$ -nAChRs was assessed through whole-cell patch-clamp experiments by applying ACh (1 mM) or Ch (10 mM) onto the soma of interneurons located in the *stratum radiatum* and at the border of the *strata radiatum/lacunosum-moleculare* of the CA1 hippocampal region. This procedure elicited  $\alpha 7$ -nAChR-mediated whole-cell currents that were sensitive to 10 nM MLA or 100 nM  $\alpha$ -Bgt (Fig. 4.1). To avoid potential contaminating effects, fast glutamatergic transmission and fast GABAergic transmission were routinely blocked with selective antagonists, as were action potentials blocked with TTX. In order to minimize desensitization of  $\alpha 7$ -nAChRs and rundown of the currents, the agonist was applied in short pulses (30 ms) every 3 min, using a

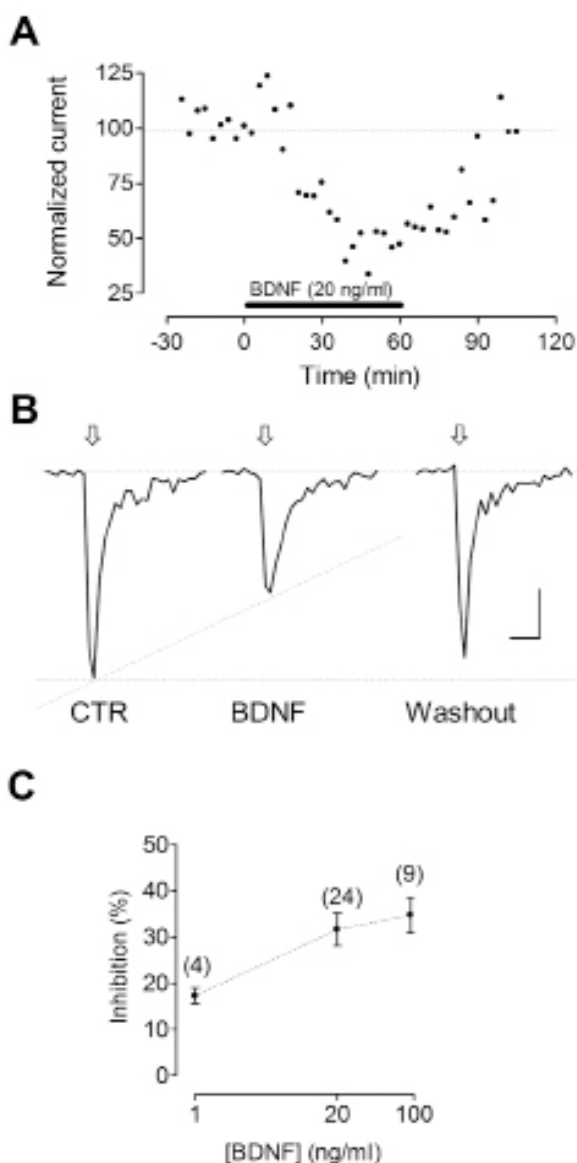




**Figure 4.1 - Patch clamp recordings of  $\alpha 7$ -nAChR-mediated currents.** Recordings were performed in interneurons located in the stratum radiatum (SR) (A) or at the border of the strata radiatum and lacunosum-moleculare (SR-LM) (B) of the CA1 region of rat hippocampal slices.  $\alpha 7$ -nAChR-mediated currents were evoked through pressure application of choline (Ch, 10 mM) or acetylcholine (ACh, 1 mM) locally onto the cell soma (C), as indicated by the arrows (D-F). The  $\alpha 7$  nAChR selective antagonists,  $\alpha$ -Bungarotoxin ( $\alpha$ -BT, 100 nM) (D) or methyllycaconitine (MLA, 10 nM) (E), completely abolished postsynaptic currents induced by 10 mM choline (Ch). F, Currents evoked by 1 mM ACh were only considered if the insensitivity to the  $\alpha 4\beta 2$  nAChR antagonist, dihydro- $\beta$ -erythroidine (dh $\beta$ E, 10  $\mu$ M), and the full blockade by  $\alpha 7$ -nAChR antagonists were observed at the end of each experiment. Scale bars: 100 ms, 100 pA (D and E); 100 ms, 50 pA (F).

pipette with 1  $\mu$ m diameter.

Stable baselines were recorded at least for 30 min before applying BDNF in the perfusion solution. BDNF (20 ng/ml) rapidly inhibited  $\alpha 7$ -nAChR-mediated currents (Fig 4.2). The inhibition reached a plateau within less than 45 min. The basal amplitudes of nicotinic responses, measured 60 min after initiating the superfusion of BDNF (20 ng/ml), were reduced in 24 of 32 cells tested by  $31.6 \pm 6.6\%$  ( $n = 24$ ,  $p < 0.001$ ) (Fig. 4.2A,B). A similar inhibition ( $38.5 \pm 7.9\%$ ) was observed when performing gramicidin-perforated patch clamp recording ( $n = 3$ ,  $p < 0.05$ ). The effect of BDNF was dependent on its final concentration in the bath solution. As shown in Figure 4.2C, whereas 1 ng/ml BDNF only attenuated  $\alpha 7$ -nAChR-mediated currents by  $17.3 \pm 1.7\%$  in 4 of 5 cells, 100 ng/ml BDNF decreased nicotinic responses by  $33.5 \pm 4.9\%$  in 9 of 14 trials, which was not



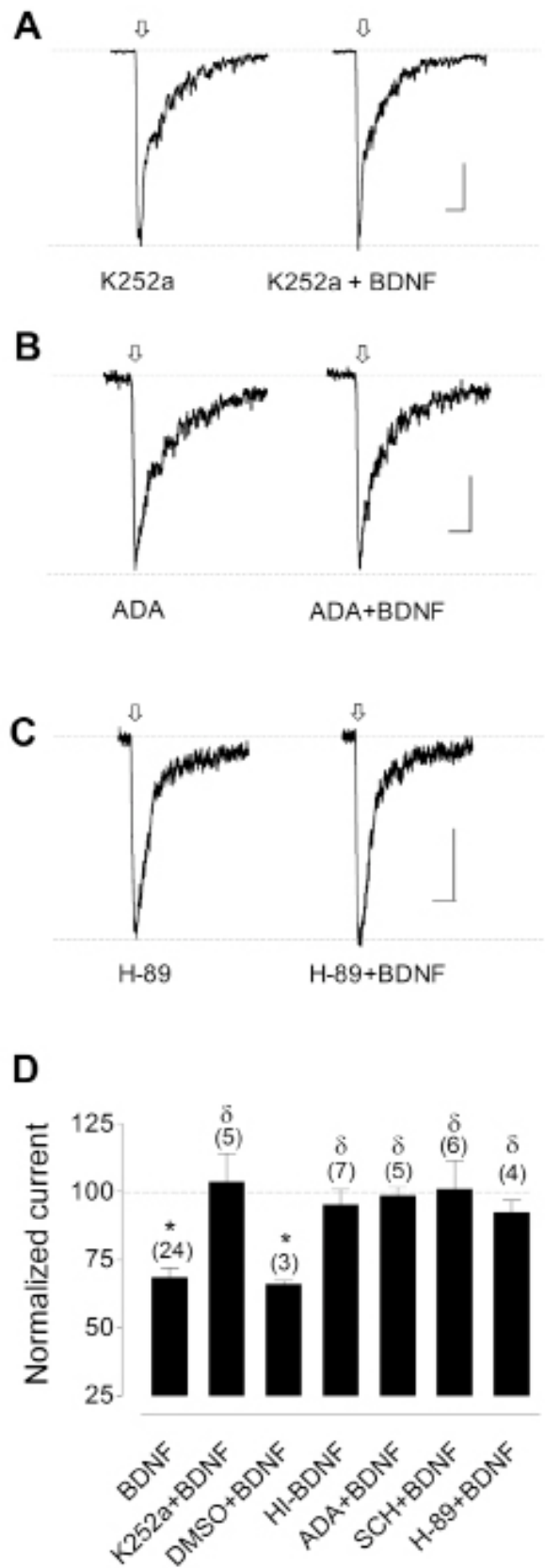
**Figure 4.2 - BDNF inhibits  $\alpha 7$ -nAChR-mediated currents in CA1 hippocampal interneurons.** A, Time course of a typical experiment showing normalized peak amplitudes of  $\alpha 7$  nAChR-mediated currents recorded in the absence of drugs, during the superfusion of 20 ng/ml BDNF (horizontal solid bar) and after a prolonged washout period, as indicated. Sample currents obtained from the same interneuron are shown in (B). Arrows indicate brief (30 ms) applications of Ch (10 mM) to the soma of interneurons. Scale bars: 100 ms, 100 pA. C, Concentration-dependent inhibition of  $\alpha 7$  nAChR-mediated responses by BDNF (1-100 ng/ml). Each point represents the percentage of inhibition achieved 60 min after initiating BDNF treatment.

significantly different from the effect produced by 20 ng/ml. The washout of BDNF (20–100 ng/ml) was attempted in 14 experiments, six of which resulted in the recovery of nicotinic responses to near baseline values ( $97.4 \pm 2.0\%$ ) within  $45.0 \pm 15.5$  min (Fig. 4.2A,B). In the remaining eight cells, the inhibitory effect of BDNF persisted for at least 1 h after the washout period was initiated. Noteworthy, the success rate of recovery during washout seemed to be correlated with the concentration of BDNF tested. Although for 20 ng/ml BDNF currents returned to baseline values in four of six experiments (66.7%), that proportion dropped to two of eight (25%) when 100 ng/ml BDNF was used. This observation, together with the fact that BDNF is a sticky molecule (Lu, 2003), suggests that the failure of  $\alpha 7$ -nAChR-mediated responses to recover might be attributable to the impossibility of obtaining a complete tissue clearance of the neurotrophin in some experiments. Superfusion of heat-inactivated BDNF (as a control for non-specific effects) did not modify the mean amplitude of the recorded currents in any of the cells tested ( $n = 7$ ) (Fig. 4.3D).

#### 4.2.2 INHIBITION OF TrkB RECEPTORS IMPAIRS BDNF-INDUCED SUPPRESSION OF $\alpha 7$ -nAChR-MEDIATED CURRENTS

Rapid actions of BDNF have been attributed to its binding to the tyrosine kinase TrkB receptors (Blum and Konnerth, 2005). To evaluate whether the TrkB receptor was involved in the inhibitory action of BDNF on  $\alpha 7$ -nAChRs, we studied the effect of this neurotrophin when the tyrosine kinase activity of Trk receptors family was inhibited by addition of the alkaloid K252a (200 nM) (Knusel and Hefti, 1992) to the intracellular solution. In

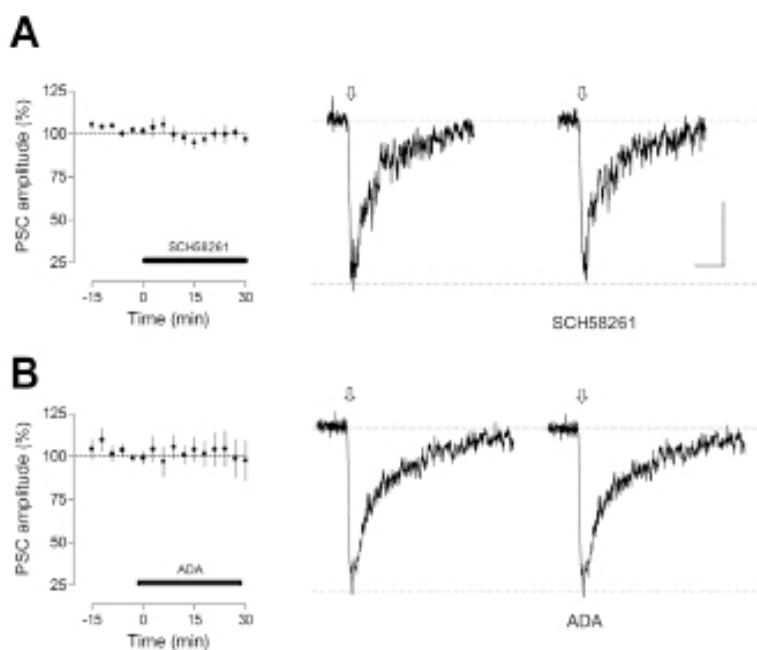
**Figure 4.3 - BDNF-induced depression of  $\alpha 7$ -nAChR-mediated currents involves a Trk-type receptor.** A, Illustrative currents showing that postsynaptic loading of the tyrosine kinase receptor inhibitor K252a (200 nM) prevented the inhibitory action of BDNF. B, Bath application of adenosine deaminase (ADA; 1 U/ml) also prevented the effect of BDNF on  $\alpha 7$  nAChRs. C, Blockade of PKA by intracellular dialysis of H-89 (1  $\mu$ M) impaired BDNF (20 ng/ml) ability to depress  $\alpha 7$ -nAChR-mediated currents. D, Summary of data, as indicated. BDNF (20 ng/ml), heat-inactivated BDNF (HI-BDNF, 20 ng/ml BDNF boiled for 30 min), ADA (1 U/ml) or adenosine A2A receptor antagonist, SCH 58261 (100 nM), were applied in the superfusion solution for at least 30 min before BDNF. K252a (200 nM), DMSO (0.1 % v/v) or H-89 (0.1-1  $\mu$ M) were included in the intracellular solution. Mean effects were quantified 60 min after BDNF application for all data. Error bars represent s.e.m. \*  $P < 0.001$  as compared to mean values before BDNF (two-tailed Student's t-test).  $\delta P < 0.05$  as compared to BDNF alone (one-way ANOVA). Scale bars: 100 ms, 100 pA.



these conditions,  $\alpha 7$ -nAChR-mediated currents were not modified by 20–100 ng/ml BDNF in any of the cells tested ( $n = 5$ ,  $p < 0.05$ ) (Fig. 4.3A).

Neuromodulation by TrkB tyrosine kinase receptors is tightly dependent on endogenous adenosine acting on  $A_{2A}$  GPCRs (Diógenes et al., 2004). Thus, it was evaluated the effect of BDNF (20 ng/ml) when the extracellular adenosine levels were reduced with adenosine deaminase (ADA, 1 U/ml) or under pharmacological blockade of adenosine  $A_{2A}$  receptors with SCH 58261 (100 nM). Despite the observation that neither SCH 58261 nor ADA affected nicotinic responses per se (Fig. 4.4), their superfusion in the bath solution prevented the inhibitory effects of BDNF in all cells tested ( $n = 5-6$ ,  $p < 0.05$ ) (Fig. 4.3B,D).

The activity of Src-family tyrosine kinases (SFKs) has been indicated as one of the mechanisms that can lead to the activation of Trk receptors by adenosine (Lee and Chao, 2001) and to the regulation of  $\alpha 7$ -nAChR-mediated currents on hippocampal interneurons (Charpantier et al., 2005). Moreover, a role for SFKs in Trk receptor signaling has also been suggested (Iwasaki et al., 1998). Thus, it was investigated whether SFKs might participate in the inhibitory effect of BDNF on  $\alpha 7$ -nAChRs. As Figure 4.5 shows, when a broad-spectrum inhibitor of SFKs, 100 nM PP2 (Berghuis et al., 2005), was loaded intracellularly, BDNF (20 ng/ml) still significantly ( $p < 0.05$ ) depressed  $\alpha 7$ -



**Fig. 4.4 -  $A_{2A}$  receptors do not influence per se the amplitude or the kinetics of  $\alpha 7$  receptor-mediated currents.** Each panel displays averaged time courses and two illustrative currents showing the absence of effect of the following pharmacological drugs on  $\alpha 7$  nAChR-mediated currents: (A) adenosine  $A_{2A}$  receptor antagonist, 100 nM SCH 58261, and (B) adenosine deaminase (1 U/ml). Arrows indicate brief (30 ms) application of ACh (1 mM) to the soma of interneurons. Currents shown on the left were obtained immediately before adding the respective drug to the bathing solution and currents on the right were evoked 60 min later. Scale bars: (a) 100 pA, 100 ms (b) 200 pA, 100 ms.

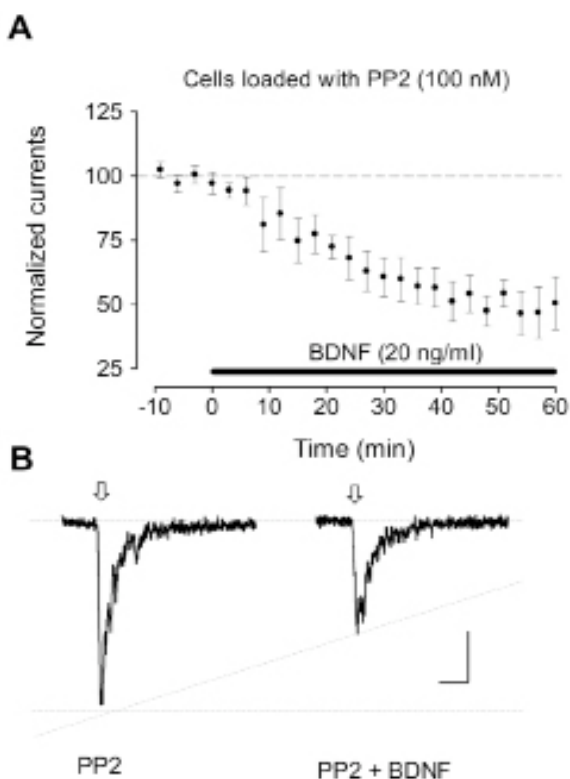
nAChR responses in four of six of cells tested by  $49.5 \pm 10.2\%$ , excluding a putative role of this family of kinases on BDNF-induced inhibition of nicotinic responses.

Adenosine  $A_{2A}$  receptors can be also coupled to the cAMP-PKA signal transduction system and it has been previously shown that PKA might play a role in the crosstalk between  $A_{2A}$  and TrkB receptors in the hippocampus (Diógenes et al., 2004). Therefore, it was investigated whether the direct inhibition of PKA with H-89 would also restrain the effect of BDNF on  $\alpha 7$ -nAChRs. In

fact, as Figure 4.3, C and D, shows, the intracellular loading of H-89 (0.1–1  $\mu$ M) prevented ( $p < 0.05$ ) the action of BDNF (20 ng/ml) in the majority of the cells tested (four of five) and in one cell attenuated it, corroborating previous evidences that cAMP-dependent

processes might regulate the rapid effects of this neurotrophin (Diógenes et al., 2004; Ji et al., 2005).

Together, the data depicted above indicate that the inhibition of  $\alpha 7$ -nAChR function by BDNF requires postsynaptic TrkB receptors with preserved tyrosine kinase activity and agree with previous evidences (Diógenes et al., 2004; Mojsilovic-Petrovic et al., 2006) that the antagonism of adenosine  $A_{2A}$  receptors inhibits activation of TrkB and/or its downstream signaling, even when cells are provided with enough extracellular BDNF to tonically activate its receptor.

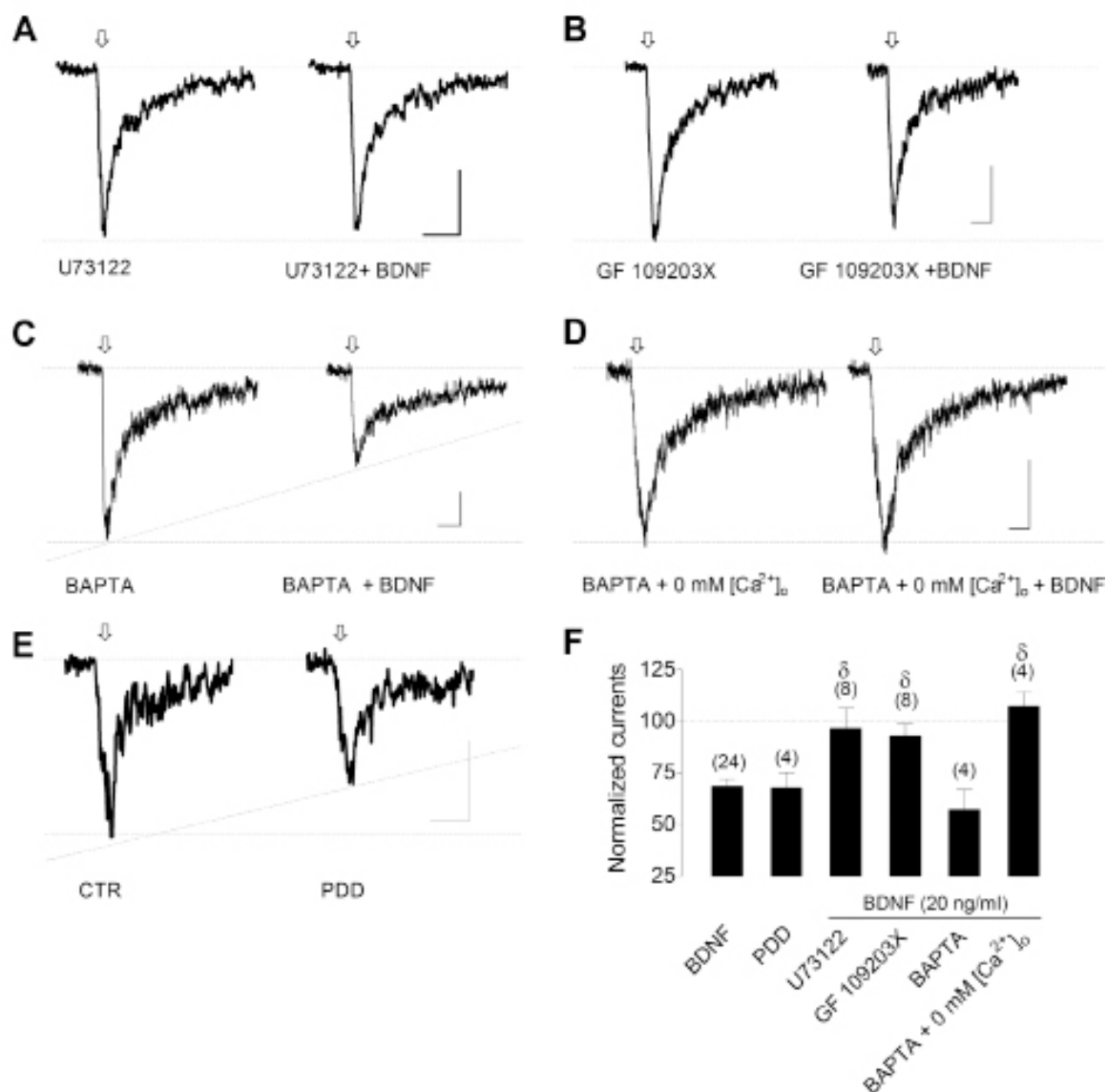


**Figure 4.5 - The inhibitory action of BDNF on  $\alpha 7$ -nAChR-mediated responses does not involve the activity of Src kinases-family.** A, Time course plots showing the effect of 20 ng/ml BDNF (horizontal solid bar) on Ch-evoked currents recorded with an intracellular solution containing a Src kinases-family inhibitor, PP2 (100 nM). Error bars represent s.e.m. Illustrative currents are shown in (B). Arrows indicate brief (30 ms) application of Ch (10 mM) to the soma of the interneuron. Scale bars: 100 ms, 100 pA.

### **4.2.3 BDNF ACTION ON $\alpha 7$ -nAChRs PROCESS REQUIRES THE PLC/PKC PATHWAY AND $\text{Ca}^{2+}$ IONS AS A COFACTOR**

The autophosphorylation of TrkB receptor tyrosine residues after BDNF binding creates independent sites for Shc and PLC- $\gamma$  (Vetter et al., 1991; Middlemas et al., 1994). Whereas the Shc site of TrkB couples the activated receptor to the Ras/MAPK pathway and the PI3K/Akt pathway, TrkB interaction with PLC- $\gamma$  is responsible for the formation of the  $\text{Ca}^{2+}$ -mobilizing second messenger  $\text{IP}_3$  and DAG, an activator of PKC. This led us to investigate whether the PLC/PKC pathway was involved in BDNF-induced inhibition of  $\alpha 7$ -nAChR. When the broad-spectrum inhibitor of PLC U73122 (5  $\mu\text{M}$ ; Tanaka et al., 1997) was included in the intracellular solution, BDNF failed to affect  $\alpha 7$ -nAChR-mediated currents in all cells tested ( $n = 8$ ) ( $p < 0.05$  compared with the effect of BDNF alone) (Fig. 4.6A,F). Dialysis of a general inhibitor of PKC isoforms, GF 109203X (2  $\mu\text{M}$ ), through the patch pipette also completely occluded the effect of BDNF (20 ng/ml) on  $\alpha 7$ -nAChR function ( $n = 8$ ,  $p < 0.05$ ) (Fig. 4.6B,F). These data together support a role for the PLC/PKC pathway in mediating BDNF-induced downregulation of  $\alpha 7$ -nAChR-mediated currents.

The PKC family comprises at least 10 isoenzymes, which can be divided into three subfamilies on the basis of their second-messenger requirements (Jaken and Parker, 2000). Conventional PKCs contain the isoforms  $\alpha$ ,  $\beta\text{I}$ ,  $\beta\text{II}$ , and  $\gamma$ , which require  $\text{Ca}^{2+}$ , DAG, and a phospholipid for activation. Novel PKCs include the  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$  isoforms and require DAG, but do not require  $\text{Ca}^{2+}$  for activation. Conversely, atypical PKCs, which include  $\zeta$  and  $\iota/\lambda$  isoforms, require neither  $\text{Ca}^{2+}$  nor DAG for activation. It was then evaluated whether the inhibitory actions of BDNF on  $\alpha 7$ -nAChR function required calcium signals to occur, in an attempt to investigate which PKC subfamily participated in that mechanism. Under these conditions, BDNF was still able to inhibit  $\alpha 7$ -nAChR-mediated currents when the fast  $\text{Ca}^{2+}$  chelator BAPTA (10 mM) was loaded intracellularly ( $43.0 \pm 9.9\%$  inhibition,  $n = 4$  of 7 cells,  $p > 0.05$ ) (Fig. 4.6C,F), the neurotrophin did not significantly ( $p > 0.05$ ) modify  $\alpha 7$ -nAChR response in any of the cells tested ( $n = 4$ ) when the intracellular dialysis of BAPTA (10 mM) was conjugated with the simultaneous removal of extracellular calcium ions (Fig. 4.6D,F). Together,



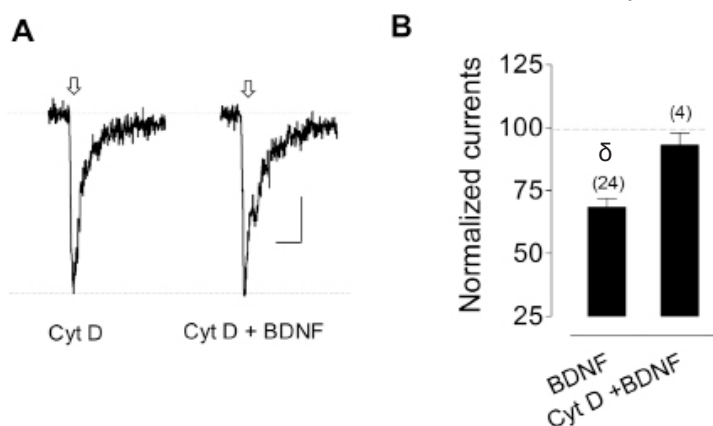
**Figure 4.6 - Signaling pathways involved in BDNF-induced inhibition of  $\alpha 7$ -nAChRs-mediated currents.** Each panel (A,B,C,D) displays two illustrative currents obtained immediately before and 60 min after adding BDNF to the bath solution (20 ng/ml). BDNF failed to modify the amplitude of nicotinic responses when the PLC blocker U73122 (5  $\mu$ M) (A) or the PKC inhibitor GF 109203X (2  $\mu$ M) (B) were dialyzed through the patch pipette. The intracellular chelation of calcium with the fast  $\text{Ca}^{2+}$  chelator BAPTA did not affect the action of BDNF on  $\alpha 7$ -nAChRs-mediated responses (C), unless extracellular  $\text{Ca}^{2+}$  was simultaneously removed (D), a condition in which the depression caused by BDNF was totally suppressed. E, Treatment with PDD (1  $\mu$ M), a PKC activator, mimicked the inhibitory effect of BDNF on  $\alpha 7$  nAChR-mediated responses. F, Histogram showing the mean effects of PDD and BDNF (20 ng/ml) on the peak amplitudes of  $\alpha 7$ -nAChRs-mediated currents after the pharmacological manipulations indicated. BDNF (20 ng/ml) and PDD were added to the bathing solution. U73122 (5  $\mu$ M) and GF 109203X (2  $\mu$ M) were included in the intracellular solution and loaded directly into the postsynaptic cell. In the experiments in which  $[\text{Ca}^{2+}]_o = 0$  mM,  $\text{CaCl}_2$  (2 mM) was substituted by  $\text{BaCl}_2$  (2 mM) in the extracellular solution. Mean effects were quantified 60 min after BDNF or PDD application for all data. Error bars represent s.e.m. Arrows indicate brief (30 ms) application of Ch (10 mM) to the soma of interneurons.  $\delta$   $P < 0.05$  as compared to the effect of BDNF alone (one-way ANOVA). Scale bars: 100 ms, 100 pA.



these data suggested, therefore, the involvement of a typical isoform of PKC (i.e., with a  $\text{Ca}^{2+}$ -binding domain) in BDNF-induced inhibition of  $\alpha 7$ -nAChRs. Notably, because intracellular  $\text{Ca}^{2+}$  chelation per se did not prevent the effect of BDNF, it is likely that  $\alpha 7$ -nAChRs might supply themselves  $\text{Ca}^{2+}$  signals that activate PKC and ultimately lead to the regulation of their function. In the next set of experiments, PKC was directly activated through superfusion of the phorbol ester phorbol-12,13-didecanoate (PDD; 1  $\mu\text{M}$ ). In such conditions,  $\alpha 7$ -nAChR responses were decreased by  $32.2 \pm 7.3$  in four of six cells, mimicking the effect of BDNF by itself (Fig. 4.6E,F).

#### 4.2.4 THE ATTENUATION OF $\alpha 7$ -nAChRs FUNCTION BY BDNF INVOLVES THE ACTIN CYTOSKELETON

The trafficking of neuronal  $\alpha 7$ -nAChRs into/from the plasma membrane depends on cytoskeleton proteins, such as actin (Shoop et al., 2000; Chang and Fischbach, 2006). Recent evidences suggesting that BDNF preserves a significant influence on the actin cytoskeleton in the mature nervous system (Rex et al., 2007) prompted us to investigate whether the attenuation of  $\alpha 7$ -nAChR function by BDNF could be regulated at that level.



**Figure 4.7 - F-actin disruption prevents the acute action of BDNF on  $\alpha 7$ -nAChRs.**

A,  $\alpha 7$ -nAChR-mediated responses evoked in interneurons loaded with the actin filament disrupter cytochalasin D (Cyt D; 5  $\mu\text{M}$ ) by local pressure application of choline (10 mM), before and during administration of BDNF (20 ng/ml). B, Barplot of mean current amplitudes for the experiments described in A. BDNF (20 ng/ml) was added to the bathing solution. Cytochalasin D was included in the intracellular solution and allowed to act directly inside the postsynaptic cell. Mean effects were quantified 60 min after BDNF. Error bars represent s.e.m. Arrows indicate brief (30 ms) application of Ch (10 mM) to the soma of interneurons.  $\delta P < 0.05$  as compared to the effect of BDNF alone (one-way ANOVA). Scale bars: 100 ms, 100 pA.

To test such possibility, it was examined the efficacy of BDNF in modulating nicotinic responses of interneurons previously loaded with the actin depolymerizing agent cytochalasin D (5  $\mu\text{M}$ ) (Cooper, 1987). Notably, BDNF (20 ng/ml) failed to significantly modify  $\alpha 7$ -nAChR-mediated currents in all cells tested under those conditions ( $n = 4$ ,  $p > 0.05$ ) (Fig. 4.7). These results suggest that the acute actions of BDNF on  $\alpha 7$ -nAChRs require the intact



actin cytoskeleton to undergo structural alterations that ultimately affect the stability, and thus the function, of  $\alpha 7$ -nAChRs in the cell membrane.

### **4.3 Discussion**

The results outlined in this chapter identified a neurotrophin gene family member, BDNF, as an acute modulator of nAChRs activity in the CNS. Under the experimental conditions used, BDNF significantly attenuated  $\alpha 7$ -nAChR-mediated currents in the vast majority of hippocampal CA1 stratum interneurons tested. The effect of BDNF on postsynaptic  $\alpha 7$ -nAChRs was dose dependent, reversible, and mediated through the activation of tyrosine kinase TrkB receptors. Additionally, it became clear that constitutively released adenosine, acting on  $A_{2A}$  receptors, was required to gate the action of BDNF. It was further demonstrated the involvement of actin cytoskeleton and PLC/PKC signaling cascade downstream of TrkB receptor activation in the attenuation of  $\alpha 7$ -nAChR function. Furthermore,  $Ca^{2+}$  is required as a co-factor. The findings presented here disclose a novel target for rapid actions of BDNF that might play important roles on synaptic transmission and plasticity in the brain.

#### **4.3.1 REGULATION OF $\alpha 7$ -nAChRs BY BDNF**

$\alpha 7$ -nAChRs-mediated currents were inhibited by BDNF in the majority of cells tested. The absence of response to BDNF verified in less than one-fourth of the interneurons might be attributable to differences in density/distribution of TrkB receptors and/or downstream signaling pathways or, less likely, to a difficult penetration of BDNF in some slices. It could also depend on the intracellular levels of cAMP, which was shown to control BDNF-induced actions in several systems (Meyer-Franke et al., 1995; Song et al., 1997; Diógenes et al., 2004; Ji et al., 2005). As described above, it was found that blockade of cAMP-mediated signaling by of PKA with H-89 reduced the effect mediated by BDNF on  $\alpha 7$ -nAChR. Thus, it is possible that the occurrence of cAMP microdomains creates conditions for isolation and/or amplification of BDNF–TrkB-mediated signal. BDNF–TrkB signaling might be selectively enhanced in active neurons or synapses with elevated  $[cAMP]_i$ . Following the same line, we can speculate that cAMP could act as an

indicator of active synapses, explaining why BDNF–TrkB signaling preferentially affects active neurons (although it was never demonstrated that neuronal activity directly increases cAMP levels).

One of the main producers of cAMP is the adenosine  $A_{2A}$  receptor (Fredholm et al., 2001). As shown here, the antagonism of adenosine  $A_{2A}$  receptors prevented the action of BDNF on  $\alpha 7$ -nAChR. Although corroborating several evidences on the tight relationship between tyrosine kinase TrkB and adenosine  $A_{2A}$  receptors, this set of results contrasts with BDNF-induced modulation of synaptic inputs to pyramidal cells, which require exogenous activation of  $A_{2A}$  receptors (Diógenes et al., 2004). It is thus possible that either adenosine levels are greater in the vicinity of interneurons or, alternatively, interneurons might exhibit an increased sensitivity for the basal levels of adenosine. In fact, both possibilities are consistent with several reports showing that extracellular adenosine levels affect interneurons in a more powerful manner than pyramidal cells (Congar et al., 1995; Fortunato et al., 1996).

It was previously reported that treatment with BDNF does not acutely modify  $\alpha 7$ -nAChRs-mediated currents in ciliary ganglion neurons, although it does upregulate  $\alpha 7$ -nAChRs expression after several days (Zhou et al., 2006). The discrepancy between that study and the present data might be explained by some differences in methodology. The most obvious difference is the type of preparation used in each study; we used freshly prepared hippocampal slices and recorded  $\alpha 7$ -nAChRs-mediated currents in interneurons, while Zhou and colleagues used dissociated ciliary ganglions in cell culture. It is well known that cells in culture develop under artificial conditions, since they are supplemented with growth factors at relatively high concentrations. In fact, the occurrence of trophic and growth factors in the intact nervous system is limited and actually cells compete for them. In this particular aspect, acutely prepared slices are closer to physiological conditions and this might be particularly important when testing the effect of trophic factors. It is also plausible that BDNF-mediated actions are cell type-specific. Ciliary ganglion neurons mediate excitatory synaptic transmission, while interneurons in CA1 *stratum radiatum* are responsible for inhibiting CA1 pyramidal cells. In this case, the acute effect of BDNF on the  $\alpha 7$ -nAChR would be confined to cells that play an inhibitory role in the circuit. Finally, in the present work,  $\alpha 7$ -nAChRs-

mediated currents were recorded in the same cell before and after applying BDNF, meaning that there are internal controls within each experiment, which is not the case for the study from Zhou and colleagues. The wide range of the peak amplitudes for  $\alpha 7$ -nAChRs-mediated currents could have masked a putative acute inhibition of BDNF on these receptors.

#### **4.3.2 INVOLVEMENT OF PLC/PKC PATHWAY ON THE REGULATION OF $\alpha 7$ -nAChR**

The results here described demonstrate here the involvement of the PLC/PKC signaling cascade downstream of TrkB receptor activation in the attenuation of  $\alpha 7$ -nAChR function. The activation of the PLC/PKC pathway by BDNF is particularly important for the control of short- and long-term brain modifications that underlie learning and memory formation (Korte et al., 1995; Gruart et al., 2007). Previous studies suggested that PKC restrained paired-pulse potentiation of  $\alpha 7$ -nAChRs in rat hippocampal interneurons (Klein and Yakel, 2005) and accelerated agonist-induced desensitization of nAChR in sympathetic ganglion neurons (Downing and Role, 1987). More recently, a study showed that muscarinic  $M_1$  acetylcholine receptors inhibit  $\alpha 7$ -nAChR-mediated currents in interneurons via PLC/PKC signaling pathway (Shen et al., 2009). Consistent with these studies is the present finding showing that activation of PKC with phorbol esters causes the inhibition of  $\alpha 7$ -nAChR-mediated currents. It would be interesting to investigate whether PKC plays an instructive or permissive role in the regulation of  $\alpha 7$ -nAChRs. Posttranslational modifications induced by the activation of several second messenger-dependent kinases have long been recognized to inhibit nAChRs from Torpedo and vertebrate muscle cells. More recently, it was shown that neuronal homomeric  $\alpha 7$ -nAChRs also constitute targets for similar mechanisms. It is tempting to speculate about the involvement of PKC in a hypothetical phosphorylation of  $\alpha 7$ -nAChRs, known to be negatively coupled to the regulation of nicotinic responses (Charpentier et al., 2005). However, predicted consensus sequences for PKC phosphorylation are absent from the intracellular domains of  $\alpha 7$  subunits, and, therefore, neuronal  $\alpha 7$ -nAChRs do not seem to constitute a potential substrate for direct PKC phosphorylation (Seguela et al., 1993; Moss et al., 1996). Nevertheless, it is possible that the acute inhibitory effect of BDNF on  $\alpha 7$ -nAChR function might involve phosphorylation/dephosphorylation

of intermediate proteins that regulate trafficking and/or clustering of the receptors. In contrast to glutamate NMDA receptors, neuronal  $\alpha 7$ -nAChRs remain active at highly negative potentials and can supply  $\text{Ca}^{2+}$  signals in these conditions. The relatively high  $\text{Ca}^{2+}$  permeability of the  $\alpha 7$ -nAChR may lead to an efficient colocalization of  $\text{Ca}^{2+}$ -binding or  $\text{Ca}^{2+}$ -dependent proteins in neurons. The results described in this chapter suggest that TrkB receptor activation leads to functional changes on the  $\alpha 7$ -nAChR that depend on  $\text{Ca}^{2+}$  influx. Because at the resting membrane potential,  $\text{Ca}^{2+}$  influx mostly occurs through  $\alpha 7$ -nAChRs, the action of BDNF here reported might play an important role on the regulation of cations influx and act to restrain excessive cell depolarization and avoid  $\text{Ca}^{2+}$ -induced excitotoxicity. In fact, the complete prevention of BDNF action on  $\alpha 7$ -nAChR responses was only achieved when intracellular  $\text{Ca}^{2+}$  chelation was combined with the replacement of external  $\text{Ca}^{2+}$  with  $\text{Ba}^{2+}$  ions, suggesting that the interactions between TrkB and  $\alpha 7$ -nAChRs probably occur in the vicinity of the plasma membrane. Such possibility is strengthened by the observation that BDNF causes a similar inhibition on  $\alpha 7$ -nAChRs function in perforated vs. whole-cell patch clamp configurations (when dialysis of cytoplasmic second messengers is prevented or when it is expected to occur at some extent, respectively).

#### **4.3.3 SHORT- AND LONG-TERM ACTIONS OF BDNF ON $\alpha 7$ -nAChRs**

It was previously reported that long-term treatment (16–72 h) with BDNF upregulates intracellular and surface pools of  $\alpha 7$ -nAChRs in subpopulations of hippocampal interneurons that mainly innervate pyramidal cells (Massey et al., 2006). However, it remains to be investigated which signaling cascades are involved in that long-term effect and whether this effect is correlated with functional modifications of nicotinic responses. Knowledge on these pathways is expected to clarify whether a common pathway downstream of TrkB receptor activation is responsible for the acute and chronic modifications of  $\alpha 7$ -nAChR function induced by BDNF. A biphasic response induced by BDNF/TrkB receptor activation would be in line with the action of neuregulin-1/ ErbB4 receptor signaling on nicotinic responses, in the sense that this system also acutely depresses  $\alpha 7$ -nAChR function and chronically enhances  $\alpha 7$ -nAChR number and function in the plasma membrane (Liu et al., 2001a; Chang and Fischbach, 2006).

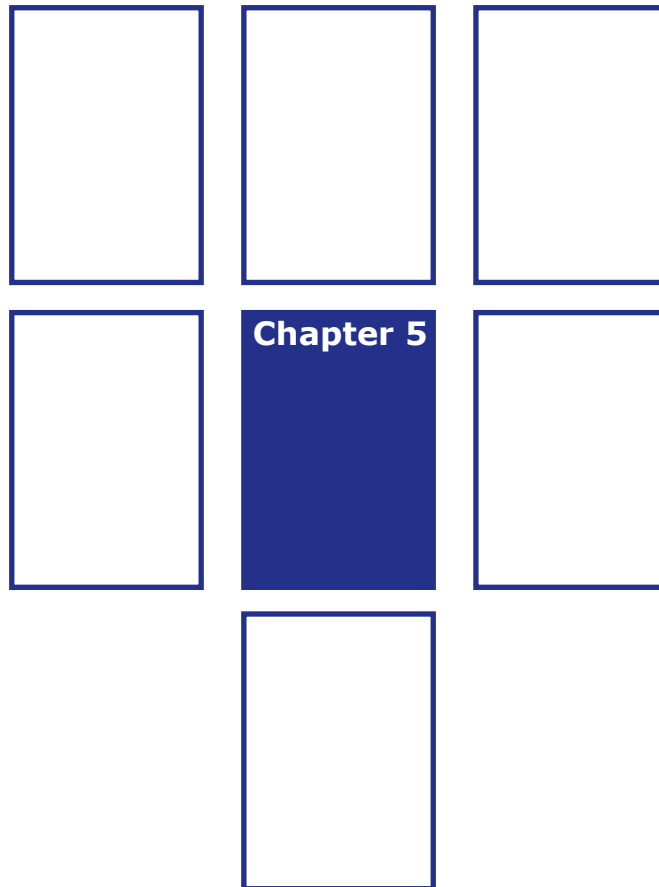
Future studies will allow determining how different tyrosine kinase receptors coordinate the regulation of  $\alpha 7$ -nAChRs and whether they interact for that purpose. Noteworthy, the actin cytoskeleton appears to play a pivotal role on both inhibitory actions of ErbB4 and TrkB receptors on  $\alpha 7$ -nAChR function.

#### **4.3.4 REGULATION OF $\alpha 7$ -nAChRS AND ITS IMPACT ON HIPPOCAMPAL CIRCUITRY**

The function of all circuits is shaped by GABAergic inhibition. In the case of *stratum radiatum* interneurons, it is thought that they primarily mediate feed-forward inhibition of pyramidal neurons. Therefore, nicotinic stimulation can shift the balance of inhibition/excitation. For instance, it has been shown that postsynaptic  $\alpha 7$ -nAChR-mediated inputs to GABAergic interneurons regulate inhibition within the hippocampal network (Jones and Yakel, 1997; Alkondon et al., 1999). In fact, a single interneuron is estimated to innervate thousands of pyramidal cells and therefore can simultaneously inhibit large populations of cells (Sik et al. 1995; Cobb et al. 1995; Buzsaki & Chrobak, 1995). Hence, the action of BDNF now described might contribute to set the background responsiveness of  $\alpha 7$ -nAChRs and should be taken into account when considering their participation in the whole neuronal network activity. In this context, because BDNF does not modify ACh-induced currents in glutamatergic neurons (Levine et al., 1998), it is plausible that the dramatic increase in BDNF secretion induced by intense stimulation of hippocampal excitatory circuits (Gartner and Staiger, 2002) might temporarily alleviate  $\alpha 7$ -nAChR-mediated inputs to interneurons that tend to oppose short- and long-term potentiation in pyramidal cells (Ji et al., 2001). Given the key role of TrkB-PLC docking site in synaptic plasticity (Gruart et al., 2007) and the reversibility of BDNF action on  $\alpha 7$ -nAChRs, the present data might also configure a mechanism involved in the adaptation to local changes in neuronal activity that occur in the hippocampus during learning and memory formation.

The results described in this chapter widen the fundamental mechanisms by which BDNF influences synaptic transmission and synaptic plasticity in the CNS. Because alterations on BDNF levels and disruption of  $\alpha 7$ -nAChR function in the hippocampus have been involved in cognitive deficits and dementia, it is expected that the link now described constitutes a target for novel pharmacological approaches for the treatment

of those disorders.



**ENDOGENOUS SIGNALLING THROUGH  $\alpha 7$ -CONTAINING NICOTINIC  
ACETYLCHOLINE RECEPTORS PROMOTES MATURATION AND INTEGRATION  
OF ADULTBORN NEURONS IN THE HIPPOCAMPUS**





## ENDOGENOUS SIGNALLING THROUGH $\alpha 7$ -CONTAINING NICOTINIC ACETYLCHOLINE RECEPTORS PROMOTES MATURATION AND INTEGRATION OF ADULTBORN NEURONS IN THE HIPPOCAMPUS

### 5.1 INTRODUCTION

The discovery of neurogenesis in the brain of adult mammals overturned the long-held dogma that adult brain has no capacity for generating new neurons. It is currently known that adult neurogenesis in the DG is essential for proper hippocampal function. Key functions for adult-born neurons include coding time and place integration (Aimone et al., 2006), spatial pattern separation (Clelland et al., 2009), reinforcement of preexisting memories (Trouche et al., 2009), and transition of memories from hippocampal to cortical circuits (Kitamura et al., 2009).

In the adult DG, new neurons are widely held to incorporate into hippocampal circuitry via a stereotypical sequence of morphological and physiological transitions, yet the mechanisms that control this process are not totally clear. Several studies indicate that the electrical activity of the existing neuronal circuitry is critical for adult neurogenesis (van Praag et al., 2005). Pioneering work on adult neurogenesis indicates that immature neurons start to express ionotropic receptors long before they are targeted by synaptic afferents, allowing them to sense transmitters in the ambience. Depolarizing GABAergic activity alters precursor proliferation and is necessary for dendritic growth of adult-born neurons (Liu et al., 2006; Ge et al., 2006). Glutamatergic activity through NMDA receptors encourages survival of adult-born neurons during a critical period when the neurons are first assimilated into behaviorally relevant networks (Tashiro et al., 2006, 2007).

The tempo of synaptic integration and maturation is significantly slower during adult neurogenesis than during embryonic and early postnatal neurogenesis (Espósito et al., 2005; Overstreet-Wadiche et al., 2006), probably due to different characteristics between adult and postnatal brains. For example, the hippocampal circuitry is much more complex in the adult brain than in the developing brain. In early postnates,  $\alpha 7$ -nAChRs contribute importantly to hippocampal development. Young hippocampal

neurons in  $\alpha 7$ KO mice have a prolonged period of GABAergic excitation because of delayed appearance of the mature chloride transporter KCC2 and extended presence of the immature chloride transporter NKCC1 (Liu et al., 2006). Furthermore,  $\alpha 7$ -nAChR signaling helps drive giant depolarizing potentials that shape network development and contribute to synaptic plasticity (Maggi et al., 2001, 2003; Le Magueresse et al., 2006). Nicotinic cholinergic input is also positioned well to influence adult neurogenesis. Early on, the neurons receive cholinergic innervation and express two major types of ionotropic nAChRs: homopentameric  $\alpha 7$ -nAChRs and heteropentameric  $\beta 2$ -containing receptors ( $\beta 2^*$ -nAChRs) (Kaneko et al., 2006; Ide et al., 2008). Substantial evidence indicates that cholinergic signaling regulates adult neurogenesis, although contributions of  $\alpha 7$ -nAChRs to adult neurogenesis have not been investigated yet.

The work described in this chapter aimed to **investigate if  $\alpha 7$ -nAChRs-mediated signaling regulates adult neurogenesis.**

## 5.2 RESULTS

The GCL is composed by a heterogeneous population of densely packed cells. Stereotaxic injection of MMLV-GFP was used to label and birthdated adultborn neurons *in vivo* in WT and  $\alpha 7$ KO young adult mice (Fig 5.1A). Once MMLV is incorporated into progenitors during mitosis, subsequent newborn cells express GFP and can be visualized in living preparations. Labeling is largely confined to neurons born within a 3-day window following virus injection (Zhao et al., 2006). Experiments were performed in hippocampal slices 3 weeks after MMLV injection, except when stated.

### 5.2.1 Nature of GABA-induced activation signaling in adult-born neurons depends on $\alpha 7$ -nAChRs

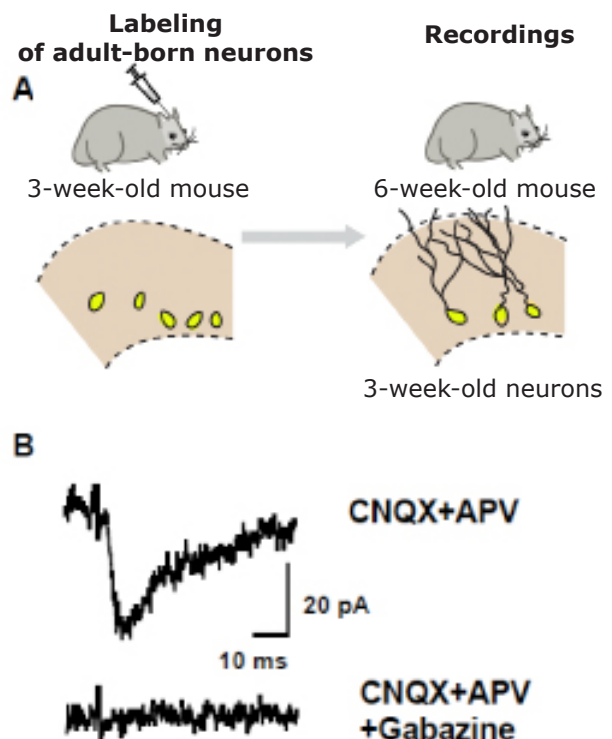
One indicator of maturation is the time during development when the  $\text{Cl}^-$  gradient acquires an equilibrium potential ( $E_{\text{Cl}}$ ) sufficiently negative to support inhibitory GABA<sub>A</sub>-mediated currents. Young hippocampal neurons in  $\alpha 7$ KO mice have a prolonged period of GABAergic excitation because of delayed appearance of the mature chloride transporter KCC2 and extended presence of the immature chloride transporter NKCC1

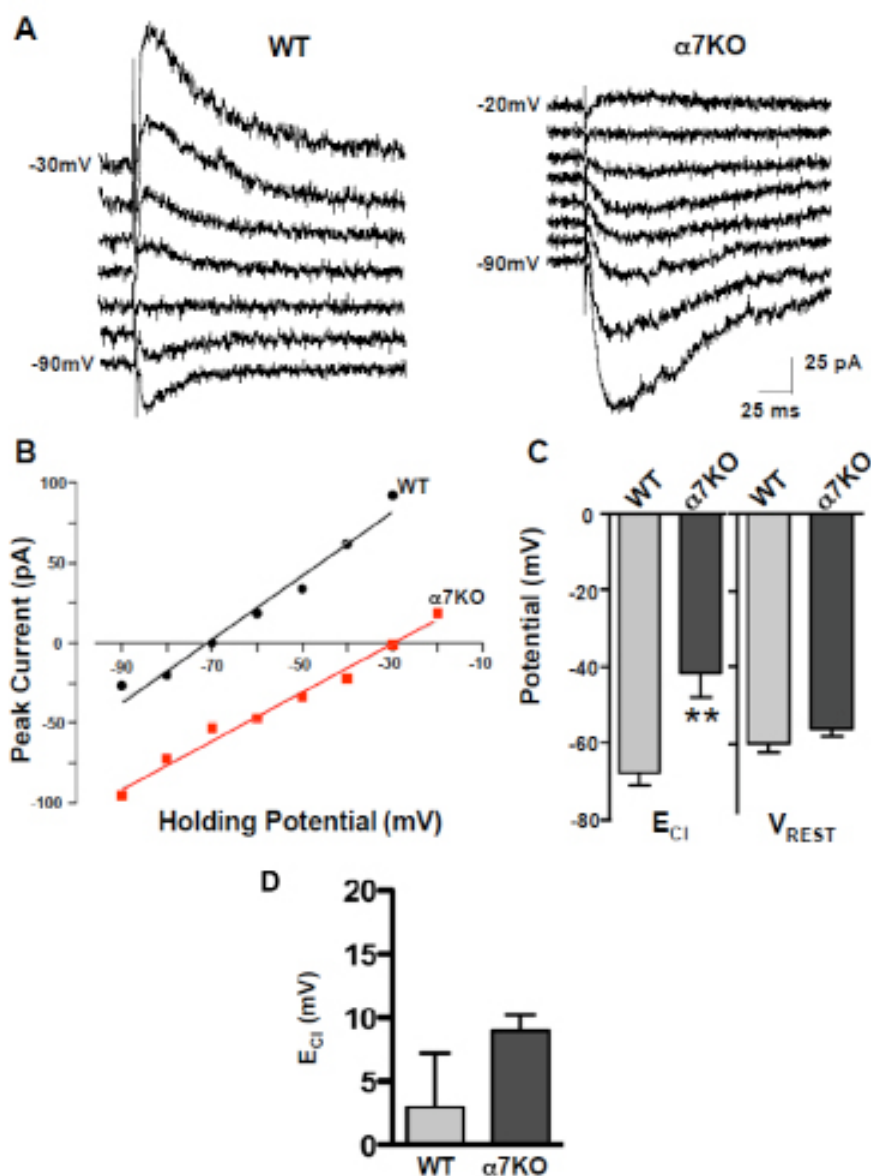
(Liu et al., 2006).

To determine if maturation of the chloride gradient is also perturbed in adult-born neurons of  $\alpha 7$ KO mice, patch-clamp recordings were performed on 3-week-old adult-born neurons (Fig 5.1A). At this stage, adult-born neurons in the wild type are expected to have a mature  $E_{Cl}$  (Esp3sito et al., 2005; Ge et al., 2006; Overstreet-Wadiche et al., 2006). Monosynaptic GABAergic postsynaptic currents (PSCs) were electrically evoked by focal stimulation of the perforant pathway in the presence of glutamate receptors antagonists CNQX (20  $\mu$ M) and APV (20  $\mu$ M). Under these conditions, all adult-born neurons from WT and  $\alpha 7$ KO animals showed pure GABAergic PSCs, since they were shown to be sensitive to the GABA<sub>A</sub> receptor blocker gabazine (10  $\mu$ M, Fig 5.1B). To overcome the problem of chloride dialysis we performed perforated patch clamp recordings with gramicidin.

Whole-cell and perforated patch configurations could be clearly distinguish in our experimental conditions, since the chloride concentration of the internal solution used was adjusted to yield a slightly positive  $E_{Cl}$  (2.7 mV) (Fig. 5.2D). By measuring the amplitude of the evoked GABAergic PSC as a function of holding potential, we were able to construct I-V plots and calculate the mean reversal potential, this being  $E_{Cl}$ . The results reveal that 3-week-old adult-born  $\alpha 7$ KO neurons retain an  $E_{Cl}$  that is significantly more positive than that of age-matched WT neurons (Fig. 5.2A-C). No change was found in

**Figure 5.1 - Recordings of GABA-mediated PSCs in adult-born neurons.** (A) Mice (3 weeks old) were stereotactically injected with MMLV-GFP to label and birthdated adult-born neurons *in vivo* in WT and  $\alpha 7$ KO young adult mice. Hippocampal slices were prepared three weeks after injection and patch clamp recordings were performed in GFP<sup>+</sup> cells. (B) Evoked PSCs in the absence (top) or presence (bottom) of gabazine to block GABA<sub>A</sub> receptors. CNQX (20  $\mu$ M) and APV (20  $\mu$ M) were present throughout to block AMPA and NMDA receptors, respectively.



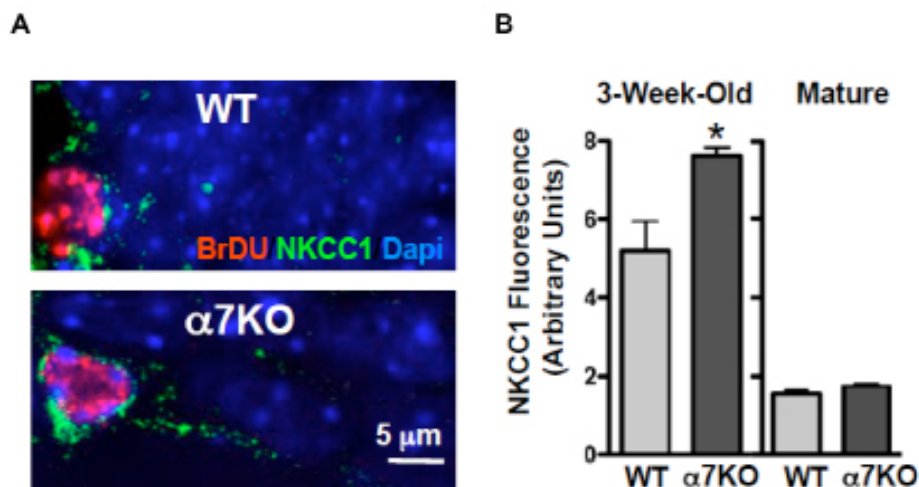


**Figure 5.2 - Delayed maturation of the chloride gradient in adultborn  $\alpha 7$ KO neurons.** (A) Superimposed perforated patch-clamp recordings of GABAergic PSCs evoked in 3-week-old adultborn WT (left) and  $\alpha 7$ KO (right) neurons at the indicated holding potentials. The neurons were labeled in vivo with MMLV-GFP and visualized in freshly prepared slices at the time of recording. (B) Peak amplitude of the evoked GABAergic PSC as a function of voltage in a WT (black) and an  $\alpha 7$ KO (red) neuron as in A. (C) Interpolated reversal potentials (left;  $E_{Cl}$ ;  $n = 6$  WT and 5  $\alpha 7$ KO) and resting membrane potentials (right;  $V_{REST}$ ;  $n = 6$  WT and 8  $\alpha 7$ KO) for WT and  $\alpha 7$ KO neurons. (D) Measurements of  $E_{Cl}$  in whole-cell patch-clamp mode from 5 WT and 3  $\alpha 7$ KO adultborn neurons at 3 weeks post-neurogenesis, identified by prior MMLV-GFP labeling in vivo. The values are in the range of that expected (2.7 mV) given the chloride concentrations present in the patch pipette and bath, and clearly different from the negative values obtained with the perforated-patch clamp recording.

the mean resting membrane potential of adult-born  $\alpha 7$ KO and WT neurons at 3 weeks of age (Fig. 5.2C). Comparing  $E_{Cl}$  to the resting membrane potential reveals that GABA<sub>A</sub> receptor activation remains depolarizing in adult-born neurons from  $\alpha 7$ KO mice after it has switched to hyperpolarizing in WT mice.

In immature neurons, the reversed chloride gradient is due to the expression of the chloride transporter (importer) NKCC1. During development, NKCC1 expression decreases and the chloride transporter (exporter) KCC2 appears. This inverts the chloride gradient, lowering  $E_{Cl}$  below threshold for action potentials and thereby rendering GABA inhibitory as required for adult function.

To compare NKCC1 levels in WT and  $\alpha 7$ KO neurons, we birthdated the neurons by BrdU injection in vivo, and then prepared slices 3 weeks later for BrdU and NKCC1 immunostaining. Three-week-old adult-born  $\alpha 7$ KO neurons in the DG displayed substantially higher levels of NKCC1 than did equivalent neurons in WT mice (Fig. 5.3 A, B). NKCC1 immunostaining in mature neurons in the outer third of the granule cell layer, which contains few adult-born neurons, revealed no significant differences between WT and  $\alpha 7$ KOs. Loss of  $\alpha 7$ -nAChR signaling, therefore, delays the reduction in NKCC1 levels in adult-born neurons but does not permanently prevent the reduction from occurring in the broader population of mature granule cells. Together, these results

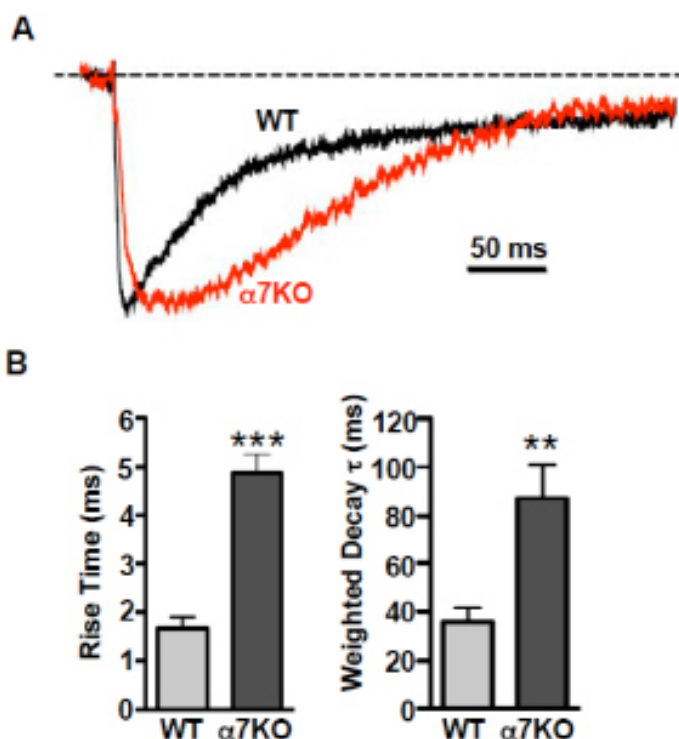


**Figure 5.3 - Inactivating the  $\alpha 7$ -nAChR gene prolongs an immature pattern of chloride transporters in the hippocampus.** (A) NKCC1 immunostaining (green) of BrdU-labeled (red) 3-week-old adultborn neurons from a WT (top) and  $\alpha 7$ KO (bottom) dentate gyrus, mounted in dapi-containing media to reveal nuclei (blue). (B) Quantification of NKCC1 levels in neurons as in D (3-Week-Old) or from neurons in the outer third of the granule cell layer (mature) from the same mice (mean  $\pm$  SEM; n = 3 animals per condition;  $\geq 10$  neurons per mouse). \* $p < 0.05$ ; \*\* $p < 0.01$ , Student's t-test.

indicate that endogenous signaling through  $\alpha 7$ -nAChRs regulates the expression of chloride transporters during development of adult born neurons and, consequently, promotes the maturation of the chloride gradient in these cells.

### 5.2.2 GABAergic PSCs retain immature temporal characteristics in the absence of $\alpha 7$ -nAChR-mediated signaling

Electrical stimulation of the granule cell layer generates GABAergic synaptic responses with different kinetics depending on the age of the cell (Hollrigel and Soltesz, 1997; Hollrigel et al., 1998; Esp3sito et al., 2005; Overstreet-Wadiche et al., 2006; Markwardt et al., 2009). Evoked GABAergic PSCs in newborn granule cells have prolonged rise and decay phases compared with PSCs in neighboring mature granule cells (although presumably a similar population of afferent fibers innervates both immature and mature newborn neurons.).



**Figure 5.4 - In the absence of  $\alpha 7$ -nAChRs, GABAergic PSCs display immature kinetics.** (A) Averaged and normalized evoked GABAergic PSCs from a WT (black) and an  $\alpha 7$ KO (red) 3-week-old adultborn neuron. (B) Quantification of rise time (left) and weighted decay ( $\tau$ ; right) of evoked GABAergic PSCs as in A (mean  $\pm$  SEM; n = 5 WT and 4  $\alpha 7$ KO). \*\*p<0.01, \*\*\*p<0.001 Student's t-test.

In the next set of experiments, the temporal characteristic of evoked GABAergic PSCs in adult-born neurons from WT and  $\alpha 7$ KO were compared. Evoked GABAergic PSCs were recorded using the perforated patch-clamp configuration on 3-week-old adult-born neurons in fresh slices previously labeled in vivo with MMLV-GFP as described above. Measuring the rise and decay kinetics of the events in adult-born neurons revealed significantly longer times in  $\alpha 7$ KO neurons compared to WT (Fig. 5.4). This suggests that adult-born  $\alpha 7$ KO neurons retain

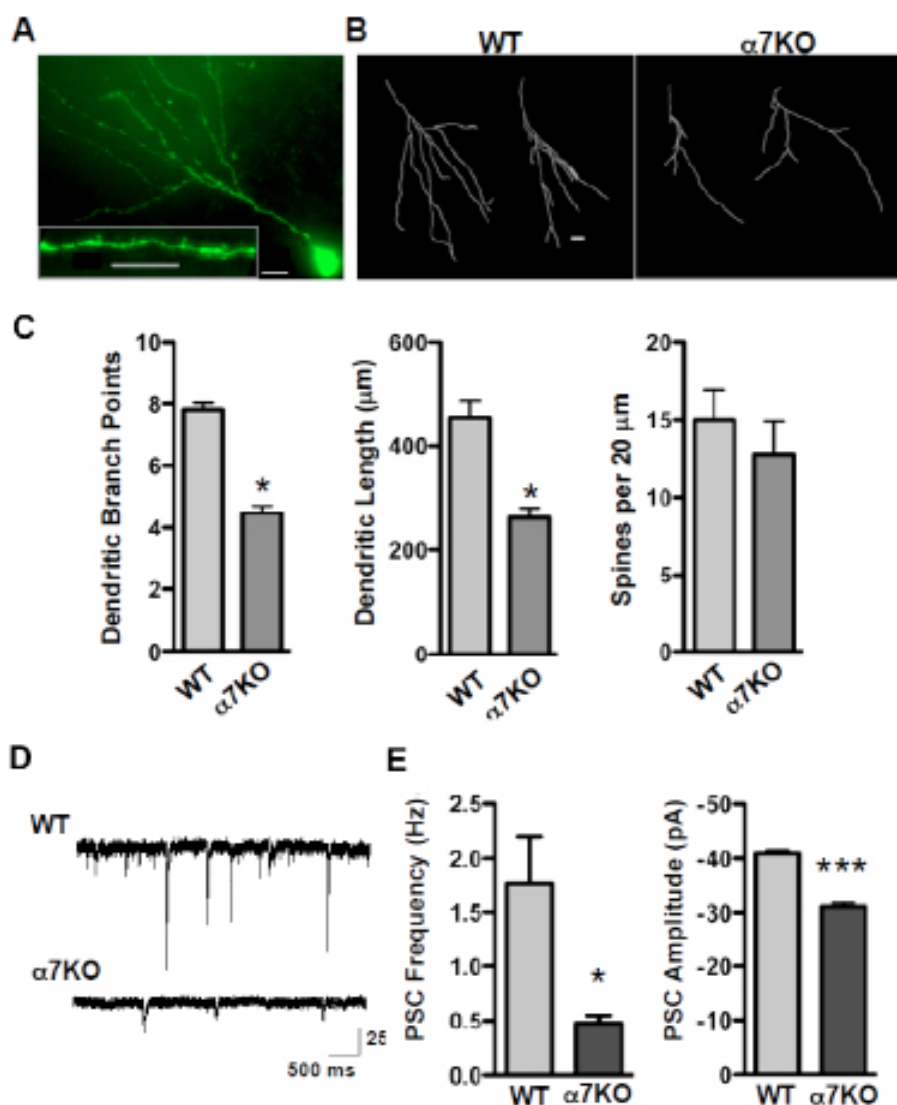
expression of immature GABA<sub>A</sub> receptor subunits over a longer developmental period than do WT.

### **5.2.3 Adult-born $\alpha$ 7KO neurons have reduced dendritic arbors and receive less synaptic activity compared with their age-matched WT counterparts**

Adult neurogenesis poses a unique challenge to new neurons in the sense that they have to be incorporated into a fully functional and active circuit. It was previously shown that depolarizing GABAergic signal is necessary for dendritic growth of adult-born neurons and integration in the network (Ge et al., 2006). Precocious conversion of ambient GABA-induced depolarization of dentate granule cells into hyperpolarization leads to defects in their synapse formation and dendritic development (Ge et al., 2006). The next set of experiments was designed to investigate if the extended period of depolarizing GABA in adult-born  $\alpha$ 7KO neurons would give them some advantage in integrating into existing circuits. Young adult WT and  $\alpha$ 7KO mice were injected with MMLV-GFP stereotaxically into the DG, and 3 weeks later taken for dendritic measurements of GFP-expressing neurons. Surprisingly, reductions in both the total dendritic length and in the number of dendritic branch points were found for adult-born  $\alpha$ 7KO neurons compared with their age-matched WT counterparts (Fig. 5.5 A-C).

Because no differences were found in the number of spines per unit length of dendrite ( $15 \pm 2$  and  $13 \pm 2$  spines/20  $\mu$ m for WT and  $\alpha$ 7KO dendrites, respectively), one can speculate that adult-born  $\alpha$ 7KO neurons are likely to have substantially fewer spines in aggregate than do WT and therefore may have proportionately fewer synapses. A reduction in synapses would account for a reduced synaptic input. To test this hypothesis, total spontaneous synaptic currents (SSCs) were recorded in adult-born neurons from age-matched WT and  $\alpha$ 7KO animals. Adult-born  $\alpha$ 7KO neurons had significantly fewer SSCs than did WT neurons, and their SSCs were smaller in size (Fig. 5.5D,E). The results indicate that in the absence of  $\alpha$ 7-nAChRs, adult-born neurons receive less synaptic activity.





**Figure 5.5 - Adultborn neurons lacking  $\alpha 7$ -nAChRs receive less synaptic inputs than normal.** (A) Deconvolved z-stack image of an adultborn granule neuron 3 weeks after the mouse was stereotactically injected with MMLV-GFP in the dentate gyrus. Digitally magnified dendritic segments (inset) were used for measurement of spine numbers. Scale bars: 10  $\mu$ m. (B) Traces of dendrites from granule neurons in WT (left) and  $\alpha 7$ KO (right) mice 3 weeks after labeling with MMLV-GFP. Scale bars: 10  $\mu$ m. (C) Quantification of dendritic complexity (left), dendritic length (middle), and spine density (right) of cells as in B (mean  $\pm$  SEM;  $n$  = 3 mice per condition with > 4 cells per animal). \* $p$  < 0.05, one-way ANOVA with Bonferroni's post-hoc test for multiple comparisons. (C) SSCs recorded in 3-week-old adultborn neurons at -80 mV holding potential, identified by GFP-labeling from MMLV-GFP injection in vivo 3 weeks earlier: WT (top);  $\alpha 7$ KO (bottom). (D) SSC frequency (left) and amplitude (right) for WT and  $\alpha 7$ KO neurons (mean  $\pm$  SEM;  $n$  = 6 WT and 5  $\alpha 7$ KO neurons). \* $p$  < 0.05, \*\*\* $p$  < 0.001, Student's t-test.

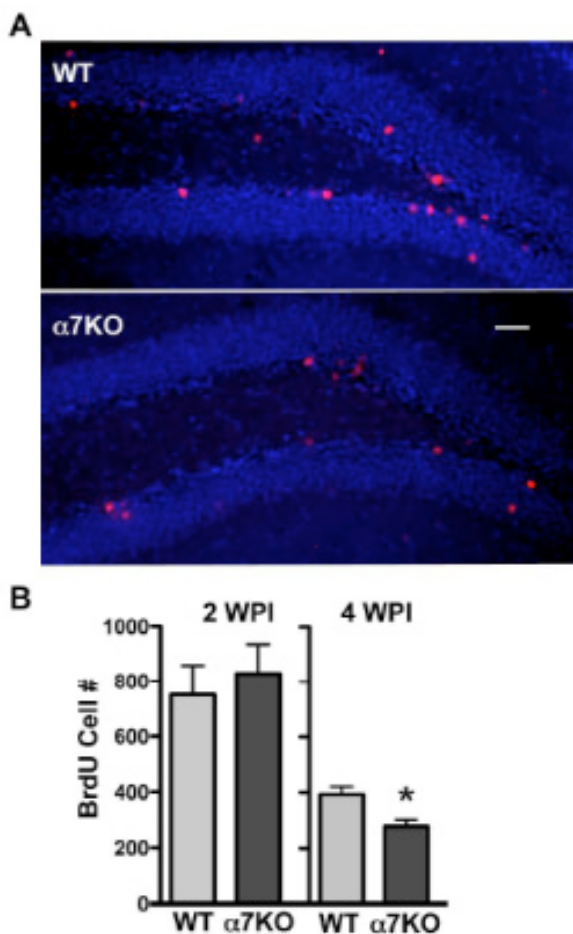


### 5.2.4 Reduced survival of adult-born $\alpha 7$ KO neurons through the critical period

A substantial fraction of newly born neurons die before they mature and the survival of new neurons is regulated in an activity-dependent manner (Kempermann et al., 1997a; Gould et al., 1999; Jessberger et al., 2005; Jessberger et al., 2007). Because adult-born  $\alpha 7$ KO neurons receive less synaptic input than adult-born WT neurons, it was decided to investigate whether the survival of new neurons is affected in the  $\alpha 7$ KO. Adult-born neurons in WT and  $\alpha 7$ KO young adult mice were birthdated by injecting BrdU and their fate was followed. Hippocampal slices were taken for BrdU immunostaining prior and after the critical period for activity-dependent survival (at 2 and 4 weeks post-injection, respectively; Tashiro et al., 2006). No difference was seen in the number of BrdU-labeled cells in the DG of WT and  $\alpha 7$ KO mice at 2 weeks (Fig. 5.6). A clear difference was found, however, at 4 weeks. At this time,  $\alpha 7$ KO mice had significantly fewer BrdU-labeled granule neurons than did 3 d window after virus injection (Zhao et al., 2006). The results indicate that, in the absence of  $\alpha 7$ -nAChRs, adult-born neurons have reduced chances of surviving through the critical period, possible due to receiving less synaptic activity.

### 5.3 DISCUSSION

It is currently known that adult neurogenesis is essential for proper



**Figure 5.6 - Absence of  $\alpha 7$ -nAChRs decreases the chance of survival for adultborn neurons during the critical period.** (A) Immunofluorescent images of the dentate gyrus showing BrdU (red) and dapi (blue) staining 4 weeks after injection of BrdU to WT (left) and  $\alpha 7$ KO (right) mice. (B) Quantification of BrdU-immunopositive cells at 2 weeks (left) and 4 weeks (right) after BrdU injection (mean  $\pm$  SEM;  $n = 4$  mice per condition). \* $p < 0.05$ , Student's t-test. Scale bar: 40  $\mu$ m.

hippocampal function. However, to participate in hippocampal-dependent behaviors, newborn neurons must functionally integrate into the pre-existing circuit. It was showed here that functional  $\alpha 7$ -nAChRs are necessary for normal survival, maturation, and integration of adult-born neurons in the DG. In  $\alpha 7$ KO mice, we find that adult-born neurons develop with truncated, less complex dendritic arbors and display GABAergic postsynaptic currents with immature kinetics. The neurons also have a prolonged period of GABAergic depolarization characteristic of an immature state. In this condition, they receive fewer spontaneous synaptic currents and are more prone to die during the critical period when adult-born neurons are normally integrated into behaviorally relevant networks.

### **5.3.1 $\alpha 7$ -nAChRs set the tempo for the maturation of GABAergic signaling in adult-born neurons**

During the first postnatal week, adult-born neurons have GABAergic synaptic input with depolarized reversal potentials (Hollrigel et al., 1998) and slow kinetics (Hollrigel and Soltesz, 1997). As previously reported (Esp3sito et al., 2005; Overstreet-Wadiche et al., 2006; Markwardt et al., 2009), at the third postnatal week, we and others found that adult-born WT neurons had reversal chloride potential and rise/decay times of GABA-PSCs characteristic of mature granule cells; in contrast, adult-born  $\alpha 7$ KO neurons showed reversal chloride potential and rise/decay times of GABA-PSCs that fall in the range of values found for immature neurons. We conclude that  $\alpha 7$ -nAChR-mediated signaling modulates the tempo of adult-born neurons development.

The initial period of depolarizing GABA is necessary for both early postnatal and adult-born neurons to develop properly and integrate into circuits (Rivera, et al., 1999; Ben-Ari, 2002; Payne et al., 2003; Ge et al., 2006). Despite being depolarizing, it is not clear if GABA acts as an excitatory or inhibitory transmitter during development. One possible justification is that GABA-induced depolarization generates  $\text{Na}^+$ -dependent action potentials or increases the probability that subsequent excitatory input trigger action potentials (Chen et al., 1996; Gao et al., 1998). In addition, GABA-induced depolarization might relieve  $\text{Mg}^{2+}$  blockade of NMDA receptors, and prone them to be activated by glutamate (Khalilov et al., 1999). This last hypothesis is consistent

with the observation that young granule cells have silent NMDA receptors in the presence of GABAR antagonists (Wadiche and Jahr, 2005). Finally, some authors suggested that depolarizing GABA inhibits cells via shunting mechanisms. In this case, nonhyperpolarizing inhibitory conductances reduce the depolarizing effects of concurrently activated dendritic excitatory PSCs via a decrease in proximal membrane resistivity and dendritic space constant (Barrett and Crill, 1974; Koch et al., 1983; Qian and Sejnowski, 1990; Staley and Mody, 1992).

The effect of depolarizing GABA in the network seems to be dependent on the temporal characteristics of GABA-mediated PSCs. It is thought that GABAergic depolarization can reduce and augment glutamatergic postsynaptic responses at short and longer latencies, respectively. The reduction is due to the shunting effect of GABA<sub>A</sub>-mediated conductance. In contrast, subthreshold glutamatergic responses summated with GABA<sub>A</sub>-mediated depolarization generated spikes if they occurred at the end of GABA<sub>A</sub> depolarization, when the shunting GABA<sub>A</sub> conductance ceased (Hollrigel et al., 1998). Theoretical simulations will help to clarify the impact of immature granule cell layers in the hippocampal circuitry.

Corroborating this line of thought are the observations that GABA<sub>A</sub>-mediated PSCs become progressively faster during maturation, when GABAergic signaling converts from depolarizing to hyperpolarizing, during maturation of adult-born neurons. As shown in this chapter, adult-born  $\alpha$ 7KO neurons, which still have a depolarized reversal potential for GABA, have significantly reduced rise/decay times for GABA<sub>A</sub>-mediated PSCs. This finding supports that adult-born  $\alpha$ 7KO neurons mature slower than neurons in the WT.

It was previously shown that differences on GABA<sub>A</sub> receptor kinetic do not depend on the complexity of the cell, but on the age of the neuron (Markwardt et al., 2009). Both the subunit composition of the receptors and the spatiotemporal characteristics of the GABA concentration transient contribute to the time course of GABA-mediated responses (Mozrzymas, 2004; Farrant and Nusser, 2005). Typically, dendritic stimulation evokes slow IPSCs (also called GABA<sub>A</sub>,slow) and stimulation near the soma evokes fast IPSCs (GABA<sub>A</sub>,fast) (Pearce, 1993; Soltesz et al., 1995; Laplagne et al., 2006, 2007). Both somatic and dendritic stimulation generate exclusively slow synaptic responses in new-born granule cells (Espósito et al., 2005; Overstreet-Wadiche et al., 2005). In

the present conditional experiments, the electrical stimulation was done in the granule cell layer, close to the cell bodies of adult-born neurons, obtaining GABA<sub>A</sub>,fast and GABA<sub>A</sub>,slow for adult-born WT neurons and adult-born  $\alpha$ 7KO neurons, respectively. This temporal difference is thought to be due to the expression of different subunits for GABA<sub>A</sub> receptors in immature and mature neurons. Early appearing forms of the GABA<sub>A</sub> receptor lack the  $\alpha$ 1 subunit and therefore lack the fast rise and decay kinetics associated with the mature form (Overstreet-Wadiche et al., 2005; Markwardt et al., 2009).

### **5.3.2 Compromised integration of adult-born in the absence of $\alpha$ 7-nAChR-mediated signaling**

Integration of new neurons into functional neuronal circuits requires proper development of synaptic inputs onto its dendrites. A period in which GABA currents are depolarizing is widely thought to be essential for promoting proper neuronal development and integration of neurons into circuits (Ben-Ari, 2002; Represa and Ben-Ari, 2005; Tozuka et al., 2005; Ge et al., 2006; Cancedda et al., 2007). Extended periods of depolarizing/excitatory GABA signaling, as found in  $\alpha$ 7KOs, could therefore be expected to correlate with increased dendritic arborization and innervation. The opposite was found: adult-born  $\alpha$ 7KO neurons have (1) shorter, less complex dendritic arbors; (2) reduced synaptic input; (3) an immature form of GABA<sub>A</sub> receptors apparently lacking  $\alpha$ 1 subunits (Overstreet- Wadiche et al., 2005). Furthermore, adult-born  $\alpha$ 7KO neurons showed an increased likelihood of dying during the critical 2–4 weeks after neurogenesis. As a result, fewer neurons are added to the adult DG, thereby compromising over time the renewal of the mossy fiber pathway. Interestingly, it was described that around 4-6 weeks of age, the adult-born neurons have a stronger synaptic plasticity than mature granule cells, as indicated by their lower threshold for the induction of LTP. Thus, it would be interesting to investigate how synaptic plasticity is affected in adult-born  $\alpha$ 7KO neurons and, if so, how it translates to behavioral tasks that depend on adult neurogenesis.

The first critical period for neuronal survival/death occurs during the third week after

neuronal birth and depends on NMDA receptors (Tashiro et al., 2006). Interestingly, this critical period is associated with a high degree of morphological change in new neurons and does not seem to require functional NMDAR. Taking into account the results described in this chapter, it is proposed that  $\alpha 7$ -nAChR-mediated signaling acts in the early stages of adult-born neurons, advancing them to some trigger point that enables them to better survive during the critical period. After this,  $\alpha 7$ -nAChR signaling drives the mechanisms that switch chloride gradient in neurons and renders GABA currents inhibitory.

The molecular mechanisms and signaling pathways that underlie  $\alpha 7$ -nAChR-dependent actions on the development of adult-born neurons are not known yet. In other systems, neurotransmitter-mediated regulation of dendritic morphology involves  $\text{Ca}^{2+}$  signaling and subsequent changes in gene expression (Borodinsky et al., 2003; Aizawa et al., 2004; Gaudillière et al., 2004). Since  $\alpha 7$ -nAChRs have a high relative permeability to  $\text{Ca}^{2+}$  (Bertrand et al., 1993; Seguela et al., 1993), they can generate  $\text{Ca}^{2+}$  events in hippocampal neurons even in the absence of detectable currents (Fayuk and Yakel, 2007; Szabo et al., 2008a, 2008b), and drive calcium-dependent gene transcription (Hu et al., 2002). Transcriptional regulation may also explain  $\alpha 7$ -nAChR-dependent changes in physiological maturation (Liu et al., 2006).

### **5.3.3 Neurogenesis and cholinergic signaling in the hippocampus**

Several studies revealed interesting differences and similarities between adult neurogenesis and embryonic/early postnatal neurogenesis. One difference is the presence of spontaneous waves of excitation known as GDPs seen in much of the developing nervous system, including in the DG (Ben Ari et al., 1989; Kasyanov et al., 2004; Overstreet-Wadiche et al., 2006). In embryonic spinal cord and early postnatal hippocampus, nicotinic activity regulates these depolarizing waves (Hanson and Landmesser, 2003; Le Magueresse et al., 2006). Consequently, GDPs may enable nicotinic activity to act ubiquitously (though indirectly) to excite large populations and coordinate their maturation. The adult DG has no comparable waves of excitation (Overstreet-Wadiche et al., 2006), and may instead rely on direct cholinergic input to guide the development and integration of adult-born neurons. Interestingly, it appears

that  $\alpha 7$ -nAChRs have some common effects in the neuronal development of the early postnatal and adult DG. It is not clear yet whether these  $\alpha 7$ -nAChR-dependent effects are cell-autonomous or occur via alterations in the circuit. For instance, the lack of  $\alpha 7$ -nAChR during embryonic development could eventually modify the architecture of the circuitry; in an extreme case, the enthorinal cortex afferents to the DG could be different in WT and  $\alpha 7$ KO animals that could justify the differences found for adult neurogenesis in the  $\alpha 7$ KO. This question could be solved by using a construct encoding an  $\alpha 7$ RNAi in WT animals to silence the activity of  $\alpha 7$ -nAChRs in a discrete population of adult-born neurons. Interestingly,  $\alpha 7$ RNAi caused dendritic arbor defects in adult-born neurons similar to those found in  $\alpha 7$ KOs (data not shown in this dissertation but see Campbell et al., 2010). These results demonstrate that  $\alpha 7$ -nAChR signaling acts in a cell-autonomous manner to regulate dendritic arborization of adult-born neurons. If physiological maturation is also a cell-autonomous process is still matter to be investigated.

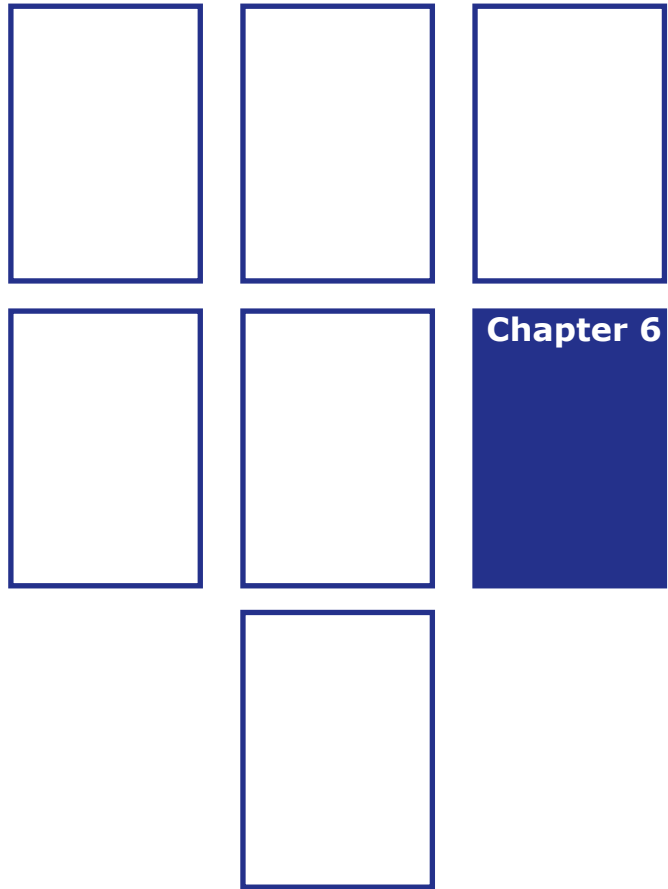
Adult-born neurons generated in young and old animals appear to have similar fates with respect to differentiation and morphological end state (Morgenstern et al., 2008; Ahlenius et al., 2009). This suggests that the results obtained here are likely to apply broadly across the population of adult-born neurons in the DG, though it should be noted that neurons born in aged adults might differ from those born in young adults in some respects. This might be particularly important when studying the role of adult neurogenesis in neurodegenerative diseases. Early deficits in Alzheimer's disease involve loss of cholinergic neurons and a diminution of cholinergic signaling (Whitehouse et al., 1982; Nordberg, 2001; Lyness et al., 2003). A $\beta$  accumulates during Alzheimer's disease in cholinergic neurons (see Gouras et al., 2010) and impairs Ch uptake and ACh release, further compromising cholinergic signaling (Mesulam, 2004). Moreover, A $\beta$  has been reported to inhibit  $\alpha 7$ -nAChR function either directly or indirectly (Wang et al., 2000a,b; Liu et al., 2001b; Pettit et al., 2001; Dougherty et al., 2003; Grassi et al., 2003; Lee and Wang, 2003; Pym et al., 2005), although A $\beta$  has also been reported to have  $\alpha 7$ -nAChR agonist activity at low concentrations (Dineley et al. 2001, 2002; Dougherty et al., 2003; Grassi et al., 2003; Wang et al., 2003). Several studies have reported specific decrements in  $\alpha 7$ - nAChRs associated with AD (Hellstrom-Lindahl et

al., 1999; Guan et al., 2000; Lee et al., 2000) (but see Rei et al., 2000). The present study predicts that the loss or blockade of  $\alpha 7$ -nAChRs in Alzheimer's disease would exacerbate the symptoms by decreasing the incorporation of adult-born neurons. Supporting this idea is the observation that donepezil, an acetylcholinesterase inhibitor approved as a drug for treatment of AD, has been shown to promote adult-born neuron survival during the critical period (Kaneko et al., 2006).

Our results identify  $\alpha 7$ -nAChRs as potential pharmacological targets for amplifying adult-born neuron integration and survival. An impediment to prescribing nicotinic agonists, however, is the observation that prolonged nicotine exposure at concentrations encountered by smokers can have detrimental effects on the survival of adult-born neurons (Abrous et al., 2002; Shingo and Kito, 2005; Scerri et al., 2006). The nicotine-mediated death of adult-born neurons occurs early in their development, whereas the beneficial effects of endogenous  $\alpha 7$ -nAChR signaling seen here become apparent 2–4 weeks after neurogenesis. Either additional nAChR subtypes are involved or the manner of receptor activation is critical. This motivates additional examination of mechanisms controlling nicotinic regulation of adult neurogenesis.







**GENERAL DISCUSSION**  
**AND**  
**FUTURE PERSPECTIVES**



## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

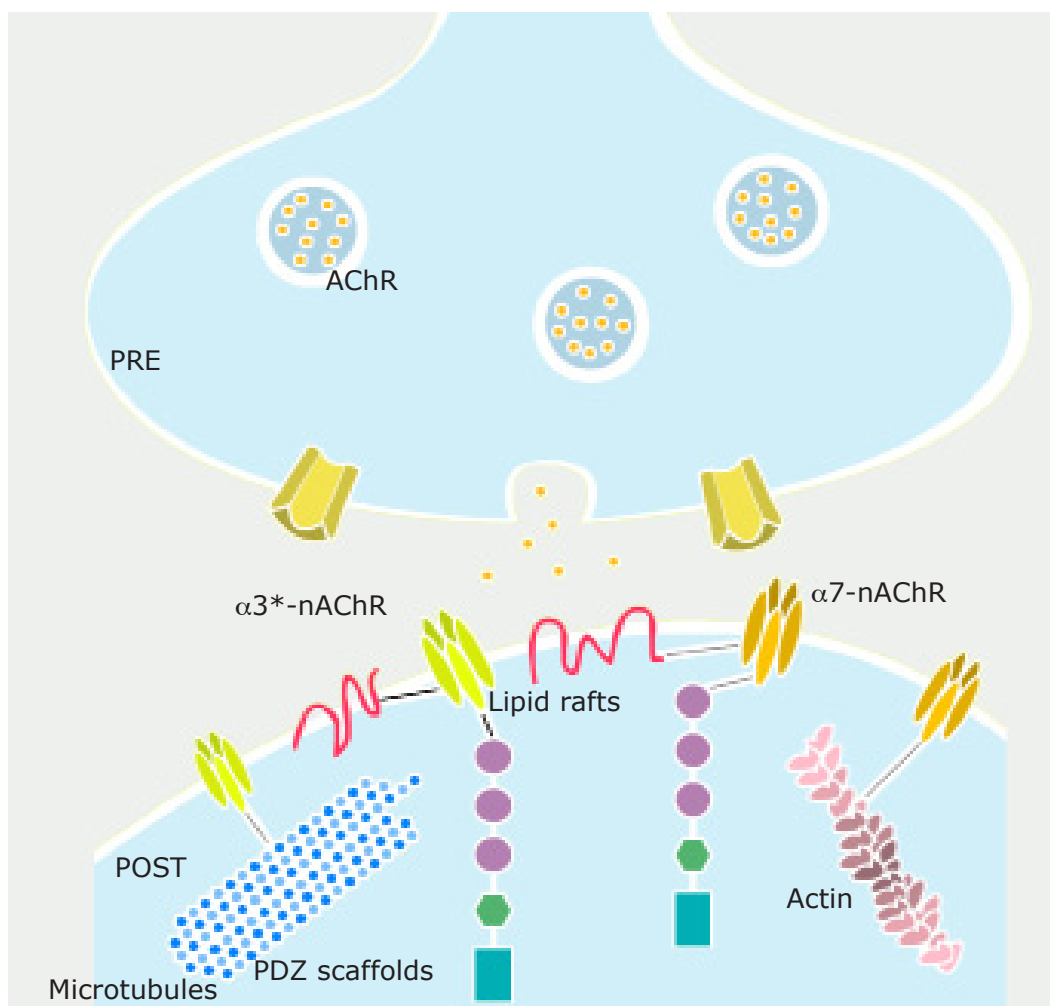
Nicotinic receptors are widely expressed throughout the nervous system, mediating synaptic transmission in the periphery and influencing electrical events in nearly every area of the mammalian brain. They enhance neurotransmitter release, modify circuit excitability, and influence synaptic plasticity. There is a huge effort trying to resolve factors and pathways that regulate the function of nAChRs. We aimed to identify some of the intracellular and extracellular molecules that regulate the trafficking and function of nAChRs, and partly clarify the role of  $\alpha 7$ -nAChRs in the hippocampus. In [Chapter 3](#) (Publication I), I investigated the lateral diffusion of  $\alpha 7$ - and  $\alpha 3^*$ -nAChRs and some intracellular mechanisms that govern this trafficking process. In this study, it was found that **cytoskeleton elements, lipids, and scaffold proteins are involved in the organization of nAChRs in the plasma membrane**. In [Chapter 4](#) (Publication II), I investigated if the function of  $\alpha 7$ -nAChRs was under control of neurotrophins, which are extracellular molecules known to play important roles in regulating synaptic strength. It was found that **BDNF acutely and reversibly inhibits  $\alpha 7$ -nAChRs-mediated currents in hippocampal interneurons, through a PLC-PKC pathway and requiring  $\text{Ca}^{2+}$  as a cofactor**. Finally, in [Chapter 5](#) (Publication III) the role of  $\alpha 7$ -nAChRs-mediated signaling in adult neurogenesis was studied. This study points out to a **critical role for  $\alpha 7$ -nAChRs in setting the tempo for maturation and integration of adult-born neurons in the existing hippocampal circuit**.

### 6.1 DYNAMIC REGULATION OF nAChRs IN THE NERVOUS SYSTEM

The specific effects of nAChRs on the nervous system critically depend on receptor location and on their associated components likely to regulate or transduce their function. We characterized the lateral diffusion of nAChRs on the cell membrane of CG neurons in culture. So far, the use of QDs to study the lateral diffusion of receptors has been limited to cell cultures due to several technical limitations discussed previously in the Methods and Materials section of this dissertation. Several factors motivated the use of cultures

of CG neurons. CGs in culture express only two classes of nAChRs, which are also found *in vivo*: the heteropentameric  $\alpha 3^*$ -nAChRs and the homopentameric  $\alpha 7$ -nAChRs (Margiotta and Berg, 1982; Vernallis et al., 1993; Chen et al., 2001). Furthermore,  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs have distinct roles on CGs neurons in culture that actually resemble their functions *in vivo*  $\alpha 3^*$ -nAChRs mediate fast excitatory neurotransmission in both sympathetic and parasympathetic autonomic ganglia (Mandelzys et al., 1995; Perry et al., 2002; Rassadi et al., 2005), while  $\alpha 7$ -nAChRs have a modulator role (Chen et al., 2001). The role of nAChRs in the hippocampus is not totally clear yet and hippocampal neurons in culture present several transmitters (but no acetylcholine), as opposed to ciliary ganglion cultures where ACh exclusively mediates synaptic transmission. We actually found here that some of the mechanisms that regulate the trafficking of  $\alpha 7$ -nAChRs are cell-type specific. It will be important in the future to expand these types of studies to brain regions where nAChRs play an important role, like the hippocampus, cortex or the ventral tegmental area (Bertrand and Dani, 2007). In CG cultures,  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs showed similar diffusion rates in the cell membrane and they can rapidly transit between synaptic and extrasynaptic areas. How receptors can enter and leave synapses remains unclear. There is so far no evidence for directed receptor transport by lateral diffusion on the somatodendritic membrane, and receptors thus reach synapses by random diffusion from the extrasynaptic membrane. There could, nevertheless, exist channels of diffusion. Furthermore, the periphery of the synapse might impose specific diffusing properties, which could create an "attractor", thus increasing the probability of trapping by the PSD (Dahan et al., 2003; Tardin et al., 2003).

I also found that the majority of the  $\alpha 3^*$ -nAChR population is immobilized in control conditions, as opposed to  $\alpha 7$ -nAChRs, which are predominantly moving in the cell membrane. I here identified two mechanisms that anchor/immobilize  $\alpha 3^*$ - but not  $\alpha 7$ -nAChRs in the membrane (Fig 6.1). The first one is a lipid rafts/cholesterol and a second one is the scaffolds of PSD95/SAP102. No anchors were identified for  $\alpha 7$ -nAChRs. This was quite surprising, since it was previously reported that cholesterol and PDZ-containing scaffolds interact with  $\alpha 7$ -nAChRs *in vivo* (Conroy et al., 2003). There are several possible explanations for these results. One is that the interactions between  $\alpha 7$ -nAChRs and cholesterol/PDZ-containing scaffolds



**Figure 6.1 - Illustration of the different cellular mechanisms constraining lateral mobility of  $\alpha 3^*$ -nAChRs vs.  $\alpha 7$ -nAChRs in synaptic space.** Immediately under the cholinergic (ACh) presynaptic terminals (Pre) is found the postsynaptic membrane (Post) containing  $\alpha 3^*$ -nAChRs (green) and  $\alpha 7$ -nAChRs (orange), as well as lipid rafts (red), overlying associated PSD-95 family scaffold proteins, nearby F-actin and microtubules, plus an unknown  $\alpha 7$ -nAChR anchor (responsible for immobile  $\alpha 7$ -nAChRs). Immobilizer links (black): prevent lateral movement unless released; slowdown links (grey): reduce receptor mobility until disrupted. No identified links are shared by the two classes of receptors.

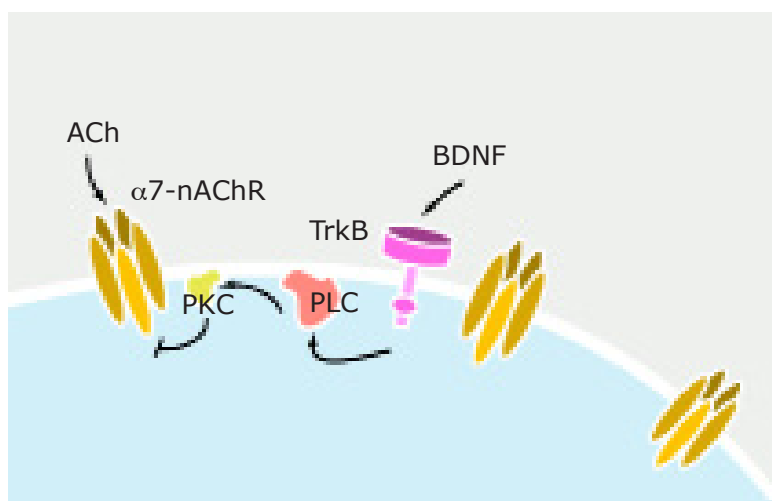
might be important for other aspects of  $\alpha 7$ -nAChR function but not for lateral diffusion. Other possibilities are that the interactions verified *in vivo* do not occur *in vitro* or occur in later stages *in vitro*. The contrasting dynamics of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs might be related to their different functions in CG neurons, where  $\alpha 3^*$ -nAChRs mediate and  $\alpha 7$ -nAChRs modulate fast synaptic transmission, respectively. The high percentage of immobile  $\alpha 3^*$ -nAChRs might reflect the stability required for mediating synaptic transmission. The importance of the  $\alpha 3$  subunit in the nervous system can be illustrated by the fact that it is the only of all the neuronal nAChR genes that produces a lethal

phenotype when deleted in inbred mice (Xu et al., 1999), most likely because this subunit is an essential component of nAChRs that mediate fast synaptic transmission in the autonomic nervous system. The different mobilities of  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs may also be related to their rates of desensitization. It was recently suggested that lateral diffusion of receptors might work as a mechanism for replacing desensitized receptors (Heine et al., 2008b). Unlike  $\alpha 3^*$ -nAChRs,  $\alpha 7$ -nAChRs are rapidly desensitized in response to agonist binding. From this perspective, the high percentage of mobile  $\alpha 7$ -nAChRs could serve as a mechanism for replacing desensitized  $\alpha 7$ -nAChRs; lateral diffusion would allow the trafficking of  $\alpha 7$ -nAChRs from exocytotic zones or to endocytic zones. In the future, the identification of the insertion and removal places of nAChRs on the cell membrane would contribute to understanding the dynamics of nAChRs. Finally, the subunit specific anchoring of nAChRs might occur at different development stages, with  $\alpha 3^*$ -nAChRs stabilization preceding  $\alpha 7$ -nAChRs in the case of CGs. Other candidates for regulating the lateral diffusion of nAChRs are cell adhesion molecules, which regulate the expression and localization of nAChRs in CGs. These unsolved questions motivate further studies on the lateral diffusion of nAChRs and their respective scaffolds. Resolving the structure of the different subunits of nAChRs, as well as identifying the subunit combinations/stoichiometries present in neurons, will help us to understand why and how different classes of nAChRs interact with different molecular partners. Knowledge on these differences will be critical for developing therapeutical strategies targeting nAChRs.

The physiological significance of lateral diffusion for transmitter receptors in the nervous system is far from clear (Gerrow and Triller, 2010). Most of our current knowledge comes from muscle nAChR subtype in the NMJ and glutamate receptors in the CNS. It is thought that lateral diffusion helps to concentrate receptors at synapses in early phases of development (Newpher and Ehlers, 2008). This mechanism seems to be retained by neurons for changing the number and/or type of receptors during short- or long-term plasticity (Heine et al., 2008b; Makino and Malinow, 2009). Moreover, lateral diffusion may constitutively substitute receptors (and other molecules) at synapses (Heine et al., 2008b). The relation between the constant flux of the constitutive elements and the homeostatic structural stability of synapses is still not understood. Some synapses

can be maintained over a time period of days, weeks and even years, though the lifetime of molecules is, at most, on the order of days. The study of lateral diffusion may contribute to our understanding the dichotomy between “synaptic plasticity” vs “synaptic stability”. Theoretical modeling of the lateral diffusion of nAChR receptors can help to clarify its impact on network activity. One of the future challenges for studying lateral diffusion of receptors is to characterize this mechanism in the intact brain.

A variety of stimuli have been shown to modulate AMPAR lateral trafficking over a wide dynamic range. For example, global glutamate application, neuronal depolarization, and long-term specific block of neuronal activity increases AMPAR mobility inside synapses (Tardin et al., 2003; Groc et al., 2004; Ehlers et al., 2007), while a local rise of intracellular calcium and a high-frequency neuronal activity both rapidly immobilize AMPARs (Borgdoff and Choquet, 2002; Heine et al., 2008b). Knowledge on the extrinsic factors that regulate the lateral diffusion of nAChRs is very limited. Molecules in the list of candidates for regulating the lateral trafficking of nAChRs should include neuregulins (Liu et al., 2001a; Chang et al., 2006) and neurotrophins (Massey et al., 2006; Chapter 4 of this dissertation). The neuregulin NRG 1 and neurotrophin BDNF seem to have similar actions on  $\alpha 7$ -nAChRs expressed by hippocampal interneurons; they can both inhibit or enhance  $\alpha 7$ -nAChRs expression on the cell membrane, due to an acute or a long-term exposure of interneurons to these molecules, respectively. Both NRG 1 and BDNF activate tyrosine kinase receptors. Whether NRG 1 and BDNF share the same downstream signaling pathway is still not known. I found that BDNF uses the PLC/PKC pathway to inhibit  $\alpha 7$ -nAChRs acutely (Fig 6.2). More recently, a proteomic study reported that  $\alpha 7$ -nAChRs and PKC interact in the cell membrane (Paulo et al., 2009), but whether PKC plays a permissive or instructive role in the regulation of  $\alpha 7$ -nAChRs is not known yet. The pathway used by NRG 1 (and its receptor Erb4) remains to be identified. Interestingly, NRG 1- and BDNF-mediated inhibition of  $\alpha 7$ -nAChRs requires a polymerized actin cytoskeleton. Another intriguing aspect is how these short- and long-term effects are related. Are they dependent on each other? Does the acute inhibition of  $\alpha 7$ -nAChRs motivate the long-term enhancement of their expression in the cell membrane? Or do short- and long-term actions use completely independent signaling pathways?



Phosphorylation of nAChRs isolated from Torpedo (Huganir et al., 1986) and vertebrate muscle cells (Miles et al., 1987) has long been recognized as an important mechanism for rapidly regulating their function. Recently it was reported that neuronal  $\alpha 7$ -nAChRs can be inhibited by direct phosphorylation of their major intracellular loop (Cho et al., 2005). Work by

**Figure 6.2 – BDNF inhibits  $\alpha 7$ -nAChR-mediated currents in CA1 hippocampal interneurons.** BDNF acts on the tyrosine kinase TrkB receptor and induces a rapid decrease of  $\alpha 7$ -nAChR-mediated responses in hippocampal interneurons of the CA1 stratum radiatum. This inhibitory effect of BDNF occurs through the PLC/PKC pathway.

Charpentier and colleagues implicate the involvement of SKFs in the process, and further suggested that other non-identified kinases might act on  $\alpha 7$ -nAChRs. Whether  $\alpha 7$ -nAChRs are targets of posttranslational modification by PKC was not determined in the present study. If it occurs, it would constitute an important regulatory mechanism for the activity of receptors at the plasma membrane and offer an additional capacity for modulation of neuronal network function.

I found that activation of adenosine  $A_{2A}$ Rs enables the action of BDNF on  $\alpha 7$ -nAChRs. The tight relation between  $A_{2A}$ Rs, TrkB receptors, and  $\alpha 7$ -nAChRs may be specially important during periods of intense neuronal activity that underlie learning and memory formation, when the levels of extracellular adenosine and BDNF are dramatically increased. Under these conditions, downstream signaling pathways activated by TrkB receptors might temporarily alleviate  $\alpha 7$ -nAChR-mediated input to interneurons, which tends to oppose short- and long-term potentiation in pyramidal cells (Ji et al., 2001). If  $\alpha 7$ -nAChRs are coincidentally activated in interneurons and pyramidal cells is not known yet. The fact that most of the cholinergic innervation in the hippocampus comes from extrahippocampal areas creates difficulties for studying cholinergic transmission in the hippocampus. Also confounding is the fact that cholinergic innervation in the hippocampus is diffused and the fact that cholinergic terminals often do not closely



contact nAChRs. One of next challenges will be studying the cholinergic system in the intact brain. A combination of tools from molecular biology with in vivo optical imaging techniques has provided new ways for noninvasive observation of the brain. For example, recently developed cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) allows monitoring of GPCR activation in the intact brain (Nguyen et al., 2010). Using these kinds of reporters will help solve important questions about cholinergic signaling in the CNS.

## **6.2 ROLE OF $\alpha 7$ -nAChRs IN THE CENTRAL NERVOUS SYSTEM – MORE UNKNOWN THAN KNOWN**

There are more questions than answers available for the physiological significance of  $\alpha 7$ -nAChRs in the CNS.  $\alpha 7$ -nAChRs and NMDARs (which have been studied intensively in the CNS) share properties that might give us clues about the role of  $\alpha 7$ -nAChRs: they are both highly permeable to  $\text{Ca}^{2+}$  and start to be expressed early on development. However, some differences between  $\alpha 7$ -nAChRs and NMDA should be closely considered.  $\alpha 7$ -nAChRs can be activated at the resting membrane potential. Since they are inward rectifying channels, they are closed at depolarized membrane potential. In contrast, NMDARs are usually activated at depolarized membrane potentials, and inactive at resting membrane potentials due to magnesium blockade. Both  $\alpha 7$ -nAChRs and NMDARs act as  $\text{Ca}^{2+}$  suppliers, though at different membrane potentials. This may justify, at least in part, the differences found in their cellular/subcellular expression and the distinct physiological roles they appear to play in the nervous system.

In humans, as well in rodents and other mammals, brain development is far from complete at birth, and many neuronal systems need to mature in response to ongoing interaction with the “ex uterus” environment. Indeed, neuronal proliferation, apoptosis, and synaptic rearrangement persist into adolescence. Cholinergic signaling continues to play an important role in post-natal brain development, which is emphasized by the brain-specific and transient expression profiles of nAChRs (Dwyer et al., 2008). In the first week after birth, the rodent hippocampus is characterized by sparse glutamatergic transmission and by immature GABAergic signaling (activation of  $\text{GABA}_A$  receptors depolarizes the cell membrane at early times).  $\alpha 7$ -nAChRs reach their maximal expression around P7 (in rodents) and play an important role during this early phase

of postnatal development. Liu and colleagues demonstrated that spontaneous nicotinic signal through  $\alpha 7$ -nAChRs is responsible for terminating GABA-induced depolarization and initiating inhibition. The concerted actions of chloride transporters and GABAergic signaling in brain development comprise an extremely wide spectrum of phenomena, and these will, of course manifest themselves in neural network function and behavior. During development, depolarizing GABAergic signaling promotes action potential activity, opening of voltage gated  $\text{Ca}^{2+}$ -channels, and activation of NMDA receptors. These responses lead to transient elevations of  $\text{Ca}^{2+}$  levels and activation of intracellular downstream signaling cascades, which are central in mediating trophic effects of GABA during development (Ben-Ari, 2002; Owens and Kriegstein, 2002). Functional and behavioral consequences of a prolonged period of depolarizing GABAergic signaling (including those found in  $\alpha 7$ KOs) are not known yet.

Recent evidence indicates that  $\alpha 7$ -nAChRs may also regulate the development of glutamatergic transmission. Exogenous application of nicotine can unsilence glutamatergic synapses in the developing hippocampus and increases glutamatergic transmission in the hippocampus (Maggi et al., 2001, 2003, 2004; Le Magueresse et al., 2006). Whether endogenous cholinergic signaling through  $\alpha 7$ -nAChRs plays a similar role is currently unknown.

After the first postnatal week, the expression of  $\alpha 7$ -nAChRs in the hippocampal formation dramatically decreases, except in the DG where the levels of  $\alpha 7$ -nAChRs remain high during adulthood (Adams et al. 2002). Interestingly, the DG is one of the two brain areas where neurogenesis occurs in adulthood. The developmental patterns of GABAergic signaling are broadly repeated during adult neurogenesis (Espósito et al., 2005; Ge et al., 2006; Laplagne et al., 2006; Tozuka et al., 2005). Indeed, it was here showed that  $\alpha 7$ -nAChRs play an important role of in the adult neurogenesis that occurs in the DG. Newly generated neurons express unique mechanisms to facilitate synaptic plasticity, which may be important for the formation of new memories (Shors et al., 2001, 2002; Rola et al., 2004; Snyder et al., 2005; Winocur et al., 2006; Aimone et al., 2009; Clelland et al., 2009; Deng et al., 2009; Deng et al., 2010). A role for  $\alpha 7$ -nAChRs in promoting the survival, maturation, and integration of adult-born neurons would be another way that  $\alpha 7$ -nAChRs contribute to hippocampal plasticity. The current work presents several lines of clear evidence that  $\alpha 7$ -nAChRs determine the tempo of

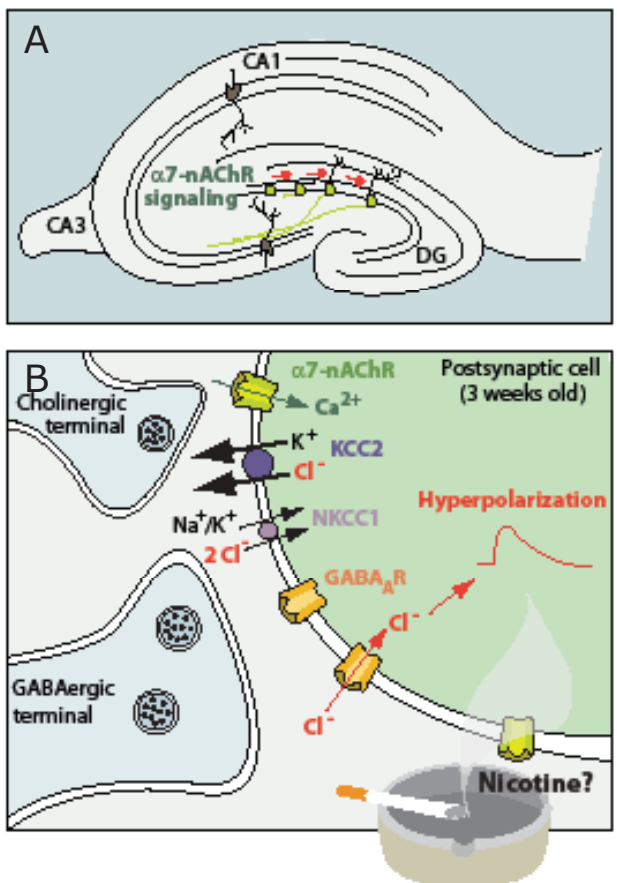
maturation and integration for adult-born neurons. The temporal characteristics of GABA<sub>A</sub>-mediated currents and the reversal potential for GABA remain immature in adult-born  $\alpha 7$ KO neurons much longer than in WT neurons (Fig 6.3).

Adult-born  $\alpha 7$ KO neurons have a reduced dendritic complexity and receive less synaptic inputs than do adult-born WT neurons. These features could explain, at least in part, the reduced survival of adult-born  $\alpha 7$ KO neurons during the critical period. Whether adult-born neurons eventually mature in the absence of  $\alpha 7$ -nAChR-mediated signaling is an important question for future investigation. For instance, 6-week old adult-born neurons in the  $\alpha 7$ KO still have a reduced dendritic complexity compared to WT (data not included in this dissertation but see Campbell et al., 2010). It would be interesting to determine whether adult-born  $\alpha 7$ KO neurons ever catch up with adult-born WT neurons in terms of physiology and the impact in the network.

The  $\alpha 7$ -nAChR-mediated downstream signaling that promotes neuronal development is an important matter for further study. As a starting point, one

**Figure 6.3 - Potential mechanisms underlying  $\alpha 7$ -nAChRs-mediated regulation of adult neurogenesis.**

(A) Sequential steps involved in generating functional and integrated new granule cells from neural progenitor in the adult hippocampus. nAChRs regulate neuronal survival and synaptic integration of new granule cells into the existing hippocampal circuitry. (B) ACh activates  $\alpha 7$ -nAChRs, leading to  $\text{Ca}^{2+}$  efflux. Other types of  $\text{Ca}^{2+}$ -permeable channel might also be activated in a voltage-independent fashion. The resulting rise of intracellular  $\text{Ca}^{2+}$  activates downstream signaling cascades, which are important for proliferation, differentiation, development dendrites and synapse formation. GABA starts being depolarizing during development due to a reversed chloride gradient, which is generated by the highly expressed chloride transporter NKCC1. Later on development, the GABA input changes from being excitatory to being inhibitory due to the increased expression of KCC2 (and decreases expression of NKCC1). How nicotine affects the development and maturation of adult-born neurons is not clear yet.



could compare the sequential gene

expression in the presence and in the absence of  $\alpha 7$ -nAChR-mediated signaling. For instance, gene arrays have become a powerful approach for comparing

complex sample RNA populations. Using array analysis, the expression profiles of WT and  $\alpha 7$ KO tissues can be compared in developmental stages of an organism or tissue. In this case, it would be easier to start the gene screening for controls and  $\alpha 7$ KOs in early stages of development and move on to adult neurogenesis in later phases. Whether the molecular mechanisms used by  $\alpha 7$ -nAChRs in embryonic and adult neurogenesis are the same is also unknown. One hypothesis is that  $\alpha 7$ -nAChRs supply  $\text{Ca}^{2+}$  signals when NMDA receptors are not prone to be activated. This could be the case for both embryonic/early postnatal neurogenesis and adult neurogenesis, when AMPA-mediated glutamatergic transmission might not be sufficient to depolarize cells and unblock NMDARs.

The activation of  $\alpha 7$ -nAChRs creates  $[\text{Ca}^{2+}]_i$  microdomains that can turn on several signaling pathways and eventually culminate in alterations of gene transcription. For this reason, it is important to study the subcellular localization and study the mechanisms that regulate the function of  $\alpha 7$ -nAChRs. In CGs neuron,  $\alpha 7$ -nAChR can transit between extrasynaptic and synaptic areas. It would be interesting to investigate if different signaling cascades are activated in synaptic and extrasynaptic areas in the future.

The release of trophic factors, including BDNF, is enhanced during periods of intense neuronal activity (Lessmann et al., 2003). It is currently thought that one function of early spontaneous network events in the postnatal brain is to promote the release of these substances. BDNF is also present in adulthood and mediates several different functions in the regulation of synaptic transmission and plasticity. It was recently shown that BDNF also controls the survival and maturation of adult-born neurons through the activation of TrkB receptors (Bergami et al., 2008). In the absence of TrkB receptors, adult-born neurons showed a reduced dendritic complexity and a lower survival rate during the critical period, which is in part similar to the phenotype that we found for adult-born  $\alpha 7$ KO neurons. These observations raise the question of whether at least some of the actions mediated by BDNF/TrkB receptor occur indirectly via modifications

on the  $\alpha 7$ -nAChRs during adult neurogenesis. Bergami and colleagues also showed that the lack of TrkB results in impaired neurogenesis-dependent long-term potentiation and in a remarkably increased anxiety-like behavior. It would be interesting to test, in the future, if the absence of the  $\alpha 7$ -nAChR causes a similar phenotype.

Quite surprisingly, the deficiency on  $\alpha 7$ -nAChRs has little impact on many mouse behaviors, since  $\alpha 7$ KO animals are apparently normal, even in old ages (Dziewczapolski et al., 2010). It was previously reported that  $\alpha 7$ KO show slightly higher levels of anxiety (Paylor et al., 1998) and have some minor defects in memory and attention (Fernandes et al., 2006). The absence of a more drastic phenotype for  $\alpha 7$ KO animals is puzzling and should be taken into account when designing new strategies for studying the role of this receptor in the CNS. A possible explanation is that other nicotinic subunits compensate for the lack of  $\alpha 7$  subunits, masking a putative stronger phenotype in the constitutive  $\alpha 7$ KO. This hypothesis is quite attractive due to the fact that  $\alpha 7$ -nAChRs are important in the first week afterbirth, when the brain has high plasticity and ability to adapt. Furthermore, all brain areas express nicotinic subunits other than  $\alpha 7$  subunits. Experiments using conditional  $\alpha 7$ KO animals (or using viral-mediated shRNA transfer to knock down the  $\alpha 7$ -nAChR) should elucidate the role of  $\alpha 7$  in animal behavior and cognitive functions such as learning and memory. Curiously, transgenic mice with a gain-of-function mutation in  $\alpha 7$  subunit neurons die shortly after birth (Orr-Urtreger et al., 2000). A likely explanation is that excessive  $\text{Ca}^{2+}$  influx through  $\alpha 7$ -nAChR at early times is lethal to the cells. This suggests that although  $\alpha 7$ -nAChRs may not be essential for normal development, their aberrant function can have detrimental effects on the developing brain.

### **6.3 THERAPEUTIC RELEVANCE OF nAChRs IN PATHOLOGY**

Abnormal nicotinic signaling has been implied in the onset of several diseases that currently have a tremendous impact in modern society. Probably the most common way of changing the signaling mediated by nAChRs in people is by their consuming nicotine, which is extracted from the tobacco leaves and is probably the most consumed drug in the whole world. Nearly one-third of adults worldwide are smokers, and the majority started the habit as adolescents. The use of tobacco is a major public health

problem worldwide and is the leading preventable cause of death in the world (WHO, 2009); it is a major cause of death from cancer, cardiovascular disease, and pulmonary disease. Cigarette smoking is also a risk factor for respiratory tract and other infections, osteoporosis, reproductive disorders, adverse postoperative events and delayed wound healing, duodenal and gastric ulcers, and diabetes. About half of those who smoke through adulthood will die from smoking-related diseases (WHO, 1997).

It was estimated that 11.7% of deaths in Portugal are attributable to smoking, based on Portuguese demographic and health statistics available from 2005 (Borges et al., 2009). Between 1995 and 2006, the total percentage of Portuguese smokers was kept constant around 25% (Precioso et al., 2009), but the percentage of smoking women increased, especially in the group of 15-24 years old, where the percentage of smokers doubled (Precioso et al., 2009). This is particularly relevant, since an ever-growing amount of evidence shows that nicotine exposure during adolescence can lead to adaptations in several brain areas that last into adulthood. Furthermore, a substantial percentage of pregnant women keep smoking during pregnancy; even when women do not smoke, they might be exposed to cigarette smoke during pregnancy. The early expression of nAChRs during fetal development gives rise to a vulnerability of the human fetus to exogenous nicotine exposure. Fetal exposure to nicotine damages the developing brain, interfering with cell replication and differentiation (Thompson et al., 2009). In longitudinal studies, it was shown that fetal tobacco exposure affects attention and impulsivity behavior (Leech et al., 1999), and can also lead to lower intelligence quotient and conduct disorder in later life (Ernst et al., 2001; Wakschlag et al., 2002).

Despite knowing the risks associated with tobacco use, the percentage of smokers that quit is still reduced mainly due to the fact that they are addicted to nicotine. Addiction is a complex behavioral phenomenon with causes and effects that range from molecular mechanisms to social interactions. Ultimately, the process of drug addiction begins with molecular interactions that alter the activity and metabolism of the neurons and consequently subverts the normal functions of the brain. Long-term exposure to an addictive drug produces neuroadaptive changes that are, in part, a homeostatic response to abnormal stimulation by the drug (Berke and Hyman, 2000; Watkins

et al., 2000; Kalivas, 2009). For example, long-term exposure to nicotine results in an increased expression of nAChRs, and that change is likely to be a homeostatic response arising from increased desensitization of nAChRs (Buisson and Bertrand, 2001). The properties of individual neurons and circuits change overtime, which leads to complex behaviors such as dependence, tolerance, sensitization, and craving (Koob et al., 1997; Nestler and Aghajanian, 1997; Laviolette SR and van der Kooy D, 2004). Addictive drugs are hypothesized to remodel circuits of the brain that normally reinforce rewarding behaviors. Many addictive drugs, including nicotine, increase dopamine levels in the mesolimbic dopamine pathway (Picciotto and Zoli, 1998; Dani and De Biasi, 2001; Bertrand and Dani, 2007), which includes dopaminergic neurons in the ventral tegmental area of the midbrain and their targets in the limbic forebrain, especially in the nucleus accumbens. It was recently shown that the dynamic regulation of BDNF synthesis and activation of TrkB-mediated signaling during ongoing cocaine use contributes to the development and maintenance of cocaine addiction. Short- and long-term actions of BDNF on nAChRs were already described (*Chapter 4*; Massey et al., 2006); whether BDNF actions contribute to nicotine addiction is a question that requires further investigation. In the last few decades, researchers have been interested in understanding the mechanisms that underlie nicotine addiction in order to develop better treatments to aid smoking cessation and the target population that has been smoking for years. It is not clear yet which nAChRs subtypes are involved in nicotine addiction. Classically, it has been considered that  $\beta 2$ -containing nicotinic receptors underlie nicotine self-administration and, by extension, tobacco addiction (Picciotto, 1998). It appears that  $\beta 2$ -containing nicotinic receptors may be more important for the initial stage of nicotine self-administration, while  $\alpha 7$ -nAChRs may be more important for continuing nicotine self-administration over a period of months (Levin et al., 2009). In addition, it was recently shown that some nicotine-induced effects are independent of nicotinic receptors (Griguoli et al. 2010), which should be taken into account in the future when searching for the mechanisms underlying nicotine addiction.

Adult-born neurons play a role in diminishing addictive behavior and reducing the incidence of relapse (Noonan et al., 2010). In showing a possible function of adult neurogenesis in drug reward and drug-context memory, these data urge continued consideration of the role of contextual cues in the treatment of addiction. We here found

that endogenous nicotinic signaling is essential to adult neurogenesis. How nicotine (or smoking) affects adult neurogenesis is not known yet. Neither is it known how adult neurogenesis influences smoking behavior.

A large number of studies suggest that the use of tobacco products among the mentally ill is significantly higher than in the general population and may be markedly higher than in normal subjects (Winterer, 2010). The incidence of heavy smoking is most frequent in subjects suffering from depression and bipolar disorder, and higher even still in schizophrenics (it should be noted, however, that the use of cigarettes in schizophrenia could have a different etiology than either depression or bipolar disorder). These observations suggest an underlying biological etiology to justify the heavy consumption of nicotine. In fact, the gene *CHRNA7* encoding the  $\alpha 7$  subunit of the nAChR, is a candidate based on an initial identification from linkage analysis of auditory evoked potential deficits observed in patients with schizophrenia (Freedman et al., 2001). It was also found that  $\alpha 7$ -nAChR transcription is altered in several ways in schizophrenic patients, suggesting that transcription-level mechanisms could account in part for the onset of the disease (Severance and Yolken, 2008). In parallel, human post-mortem tissues from schizophrenic patients show a significant decrease in BDNF concentrations in cortical areas and in the hippocampus, and polymorphisms in the BDNF gene are associated with schizophrenia. If  $\alpha 7$ -nAChR deficits are thereby considered as epiphenomena of underlying neurotrophin disorganization is not known yet. More recently, it was also suggested that regulation of adult hippocampal neurogenesis represents a promising approach for treating and perhaps preventing mental illness (DeCarolis and Eisch, 2010).

The most common form of degenerative dementia is AD, which affects more than 15 million people worldwide and grows as the proportion of elderly persons increases (Palmer, 2002). Amyloid plaques arising from deposition of A $\beta$  peptides and neurofibrillary tangles arising from aggregates of hyperphosphorylated tau protein are hallmarks of AD, which is characterized by progressive cognitive dysfunction, particularly in learning and memory. AD advances to affect limbic structures, subcortical nuclei, and cortical regions, and in that way influences multiple neurotransmitter systems. The most well appreciated neuronal loss, however, is in the cholinergic system, particularly the basal



forebrain cholinergic system comprised of the medial septal nucleus, the horizontal and vertical diagonal bands of Broca, and the nucleus basalis of Meynert. The decline of cortical cholinergic activity as measured in postmortem brains correlates with the severity of AD symptoms and with the intellectual deterioration observed in life. As AD symptoms worsen, cholinergic neurons are progressively lost and the number of nAChRs declines, particularly in the hippocampus and cortex. nAChRs have been implicated in AD, in part because significant losses in radioligand binding sites corresponding to nAChRs have been consistently observed at autopsy in a number of neocortical areas and in the hippocampi of patients with AD (Burghaus et al., 2000; Nordberg, 2001). Recently, it was reported that forebrain cholinergic neurons express an unusual combination of  $\alpha 7\beta 2$ -nAChRs, which are particularly sensitive to functional inhibition by a pathologically relevant concentration of A $\beta$  (Liu et al., 2009). It would be interesting to study the mechanisms behind the action of A $\beta$  on  $\alpha 7\beta 2$ -nAChRs. Does A $\beta$  compete with ACh-binding sites and contribute to the inhibition/desensitization of  $\alpha 7\beta 2$ -nAChRs? Does A $\beta$  cause posttranslational modifications of  $\alpha 7\beta 2$ -nAChRs that cause the rundown of ACh-mediated currents? Does A $\beta$  cause changes in the trafficking of  $\alpha 7\beta 2$ -nAChRs? Several reports indicate that alterations in trafficking of receptors likely to contribute to cause diseases such as depression, schizophrenia, and addiction (Nader et al., 2006; Witkin et al., 2007; Kessler et al., 2009). Detailed studies on the trafficking of nAChRs in models of Alzheimer's disease should clarify if the dynamics of these receptors is changed and contributes to the onset of the disease.

Evidence that BDNF levels are decreased in Alzheimer's disease patients has been growing in the last decade (Fumagalli et al., 2006). Whether the loss of cholinergic signaling in Alzheimer's disease is directly related to reduced levels of neurotrophins has never been investigated. A recent article showed that gene therapy involving BDNF delivery improves cell signaling and restores learning and memory in several animal models Alzheimer's disease (Nagahara et al., 2009). These studies provide support for exploring the clinical translation of BDNF delivery as a potential therapy for Alzheimer's disease. Early and long lasting actions of BDNF on nicotinic signaling (Chapter 4; Massey et al., 2006), and the impact of nicotinic transmission on adult neurogenesis (Chapter 5), should be taking into account when designing such therapies.

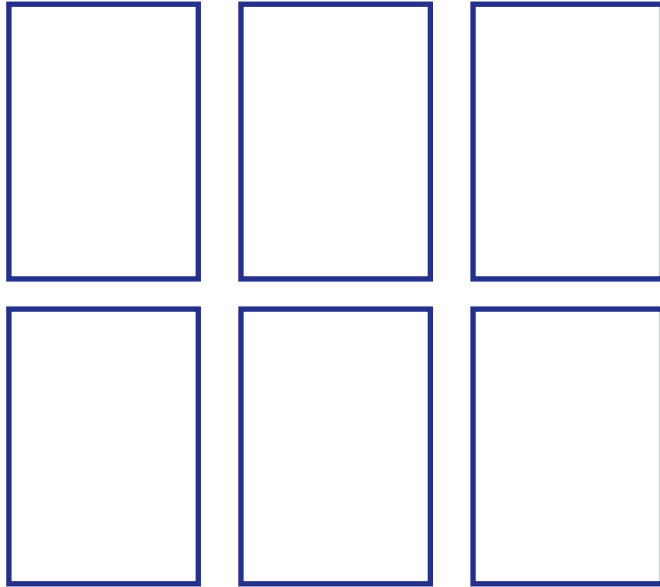
## 6.4 PERSPECTIVE

Some significant progress has been made during this project, and clear future directions for the continuation of this research have emerged.

- I. Chapter 3 - nAChR lateral mobility is determined by mechanisms that are domain-specific, receptor subtype-dependent, and cell-type constrained. The nature of nAChR movement restraints was different for  $\alpha 3^*$ - and  $\alpha 7$ -nAChRs; lipid rafts, PDZ-containing scaffolds, microtubules, and actin filaments differentially affected their mobility. The outcome is a system that could tailor nicotinic signaling capabilities to specific needs of individual locations.
- II. Chapter 4 -  $\alpha 7$ -nAChR was found to be a target for rapid actions of BDNF. BDNF rapidly reduced the amplitude of  $\alpha 7$ -nAChR mediated currents when applied in the perfusion solution. This effect was dependent on phospholipase C/protein kinase C signaling pathway and required  $\text{Ca}^{2+}$  as a cofactor. The present findings disclose important roles for  $\alpha 7$ -nAChR and BDNF that should be taken into account when looking at the actions of these players on synaptic transmission and plasticity in the brain.
- III. Chapter 5 -  $\alpha 7$ -nAChR contributes to the survival, maturation and integration of adult-born neurons in the network. This evidence points to a critical role of  $\alpha 7$ -nAChR in the fate of newborn dentate granule neurons.







## **Chapter 7**

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